THE RELATIVE VITAMIN A POTENCY OF CAROTENE FED IN BUTTER FAT AND COTTONSEED OIL[1, 2]

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TWO CHARTS

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The amounts of vitamin activity due to the carotene and vitamin A respectively of butter fat cannot be determined with precision at present. In the work that has been reported on this subject (Shrewsbury and Kraybill, '33; Baumann and Steenbock, '33; Baumann, Steenbock, Beeson and Rupel, '34; Booth, Kon, Dann and Moore, '33; and Gillam, '34) certain assumptions have been made with respect to the relative potency of equal quantities of carotene and vitamin A. Even though we know the amounts of carotene and vitamin A in butter fat, in order to determine the vitamin potency of each of these constituents, it is necessary to know their relative vitamin potency when fed in butter fat.

Dutcher, Harris, Hartzler and Guerrant ('34) found that the presence of mineral oil lowered the vitamin potency of carotene but did not affect adversely the vitamin potency of cod liver oil and a cod liver oil concentrate. The adverse effect of mineral oil in the case of carotene was explained on the basis of carotene excretion from the body in the unabsorbed oil. Lathbury and Greenwood ('34) report that different batches of arachis oil and coconut oil vary in their

[1] Wesson oil, a refined cottonseed oil.

[2] Presented at the meetings of the American Chemical Society, April, 1935, New York City.

suitability as carriers for the biological testing of vitamin A or carotene and that the suitability of any oil is not entirely dependent on the stability of the dissolved substances in the oil. They make the interesting observation that some samples of arachis oil are better than coconut oil while others are not so good as solvents for testing carotene or vitamin A. They point out further that it is not unlikely that certain oils contain a factor necessary to supplement highly purified concentrates of vitamin A and carotene but that this factor is apparently not present in oils of any one kind.

Dyer, Key and Coward ('34) found that a solution of international standard carotene in arachis oil had from five to six times the potency of similar solutions in hydrogenated cottonseed oil and that one sample of cod liver oil dissolved in coconut oil had three times the potency as when dissolved in hydrogenated cottonseed oil.

Utilization of carotene[3] *from charcoal decolorized butter fat*

In a previous paper (Shrewsbury and Kraybill, '33) from this laboratory we have shown that butter fat treated with charcoal loses its color, vitamin A activity and antioxidants. Carotene added to this butter fat containing hydroquinone is quite stable.

Experiments in which carotene was fed in charcoal decolorized butter fat indicated that at least 2 micrograms per day were required for one Sherman unit (3 gm. average gain per week for 8 weeks). This amount is four times the amount of carotene administered in cottonseed oil found to be necessary for one Sherman unit. This was true even when the dose of carotene and butter fat was prepared fresh daily, or when hydroquinone was added to the preparation to prevent oxidation of carotene.

[3] The carotene used in these studies was obtained from the S.M.A. Corporation, Cleveland, Ohio. We are indebted to Doctors Miller, Zscheile and Hogness of the chemistry department of The University of Chicago for the following analysis of the carotene: Beta carotene, 81.0 per cent; alpha carotene, 13.0 per cent; colorless impurities, 6.0 per cent.

Since the color of charcoal decolorized butter fat containing added carotene and hydroquinone was found to be stable by spectrophotometric measurements the question arose as to whether hydroquinone affected the utilization of carotene in the animal body.

Several samples of cottonseed oil containing from 2.0 to 100 mg. of hydroquinone per 100 cc. of oil were prepared. Carotene was added to all of these in an amount that would furnish 1 microgram in 5 drops of cottonseed oil. These materials were fed to rats depleted of their vitamin A stores.

TABLE 1

The effect of hydroquinone on the utilization of carotene in cottonseed oil

MILLIGRAMS HYDROQUINONE PER 100 GM. COTTONSEED OIL	CAROTENE IN DAILY DOSE MICROGRAMS	NUMBER OF ANIMALS STARTED ON EXPERIMENT	NUMBER OF SURVIVORS	SEX DISTRIBUTION	AVERAGE WEEKLY GAIN IN GRAMS AT END OF			
					Fourth week	Fifth week	Sixth week	Eighth week
				♂ ♀				
None	1.0	5	5	3—2	7.5	7.4	6.9	6.3
2.0	1.0	5	5	4—1	7.2	7.6	7.3	6.9
10.0	1.0	4	3	1—3	9.2	8.0	8.1	9.5
25.0	1.0	4	4	1—3	8.5	8.8	8.2	7.7
50.0	1.0	4	4	1—3	8.2	8.4	8.1	7.3
100.0	1.0	3	3	2—1	5.7	5.1	5.0	5.8

The experimental technic used in these experiments was the same as previously described (Shrewsbury and Kraybill, '33). The results are given in table 1.

There is no significant interference with the utilization of carotene by hydroquinone.

Decolorization of butter fat with Lloyd's reagent

A number of absorbents were examined with the hope of finding one that would remove the vitamin A activity and leave the antioxidants undisturbed. Lloyd's reagent proved to be quite efficient in this respect.

An absorption tube 6 inches long by 1 inch in diameter was filled to about three-fourths capacity with Lloyd's reagent. A plug of cotton was placed in the bottom of the tube

and the Lloyd's reagent packed in firmly by introducing small quantities at a time and tapping the tube. This tube was attached to a side neck suction flask. The butter was melted and drawn through the column with the aid of suction. The rate of flow through the Lloyd's reagent was slow and it was necessary to keep the material warm (below 50°C.) to prevent solidification of the butter. The butter fat was passed through the column twice in most cases. This treatment removed practically all of the color and the vitamin A content of the butter fat (see chart for vitamin A activity). However,

TABLE 2

Stability of carotene in butter fat decolorized with Lloyd's reagent. Materials stored at 40°C. Expressed as milligrams of carotene per 100 gm. of fat

SAMPLE	FRESH SAMPLE	SAMPLE AFTER STORAGE	STORAGE TIME IN DAYS
1	0.65	0.63	14
2	0.55	0.63	20
3	0.53	0.55	16
4	0.67	0.55	16
5	1.13	1.00	12
6	1.01	0.92	56
7[1]	0.47	0.47±0.005	60
8	0.49	0.54	10

[1] Sample number 7 was prepared by adding carotene equivalent to 0.5 mg. per 100 gm. of butter fat. We are indebted to Doctors Miller, Zscheile and Hogness of the chemistry department of The University of Chicago for making this analysis by spectro-photoelectric methods. (Plant Physiology, '34, vol. 9, p. 681.)

unlike butter fat treated with charcoal that decolorized with Lloyd's reagent retained antioxidant properties. The stability of carotene added to butter fat decolorized with Lloyd's reagent is demonstrated by the results of spectrophotometric readings given in table 2.

Because of the slow rate of flow of butter fat through the Lloyd's reagent column another method of decolorization was tried. Fifty grams of Lloyd's reagent was allowed to stand in contact with 200 gm. of melted butter fat in a stoppered Erlenmeyer flask at 50°C. for about 8 hours. The mixture was agitated from time to time. The Lloyd's reagent was

filtered off through a Buchner funnel fitted with a qualitative filter paper. The funnel was kept warm with a hot water coil. For complete decolorization it was usually necessary to repeat the treatment with Lloyd's reagent. Butter fat prepared in this manner contained no appreciable color or viamin A potency but retained antioxidant properties.

Extraction of the Lloyd's reagent used to decolorize butter fat, with ether followed by alcohol yields a part of the color. In one experiment 11.63 per cent of the original coloring matter of butter was recovered by ether extraction and 18.15 per cent by extraction with 95 per cent alcohol. A total of 39.78 per cent of the color was recovered.

Extraction of the charcoal used to decolorize butter with ether and alcohol did not effect recovery of any of the color.

It seems that in the case of charcoal the reaction completely destroys or removes the carotene and antioxidants presumably by oxidation while apparently only slight losses occur when Lloyd's reagent is used. This may explain the antioxidant properties of butter prepared by treatment with Lloyd's reagent and the lack of antioxidant properties in charcoal decolorized butter.

Utilization of carotene from Lloyd's reagent decolorized butter fat

Since carotene added to butter fat decolorized with Lloyd's reagent is quite stable this material was used to study the utilization of carotene from butter fat. Varying amounts of carotene (from 0.5 to 4.0 micrograms) were fed to rats in from 50 to 400 mg. of decolorized butter fat and also in similar quantities of cottonseed oil. The feeding samples were prepared fresh every 2 weeks throughout the feeding period of 8 weeks. The data are given in charts 1 and 2.

These data demonstrate that there is a considerable difference in the vitamin potency of carotene when fed in cottonseed oil and when fed in butter fat. A daily dose of 0.5 microgram of carotene in cottonseed oil per rat produced approximately 3 gm. gain per week for 8 weeks. The mortality was low; eleven of the thirteen rats starting in the

experiment survived. An increase in the carotene dosage caused an increase in gain roughly proportional to the amounts administered. The cottonseed oil contained negligible quantities of vitamin A. Twenty drops of plain cottonseed oil per rat daily furnished no better protection against vitamin A deficiency than 5 drops. The survival period,

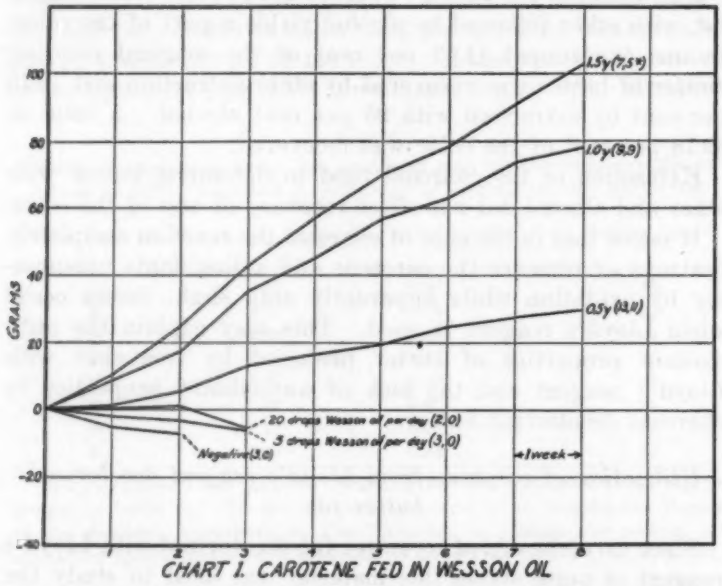

CHART 1. CAROTENE FED IN WESSON OIL

Chart 1 Figures on curves indicate in micrograms the daily dose of carotene per rat. Figures in parentheses indicate the number of animals started in the experiment and the survivors. * Two animals removed to conserve carotene.

however, was slightly longer than obtained with the negative controls.

One-half microgram of carotene per rat per day fed in decolorized butter fat produced practically no gain in weight and the mortality was high. Only one animal of fourteen finished the experiment alive. When the dosage was increased to 1.0 microgram per rat per day the growth was nearly equal to that produced by 0.5 microgram of carotene fed in cottonseed oil but the mortality was high. Not until 2.0 micrograms

of carotene per rat per day were fed was the mortality reduced to a point comparable to the mortality on 0.5 microgram of carotene fed in cottonseed oil. Growth at this level was more rapid. Decolorized butter fat had little or no vitamin A activity. As much as 1.0 gm. of this butter fat per rat per day maintained vitamin A depleted rats only slightly longer than the unsupplemented basal diet.

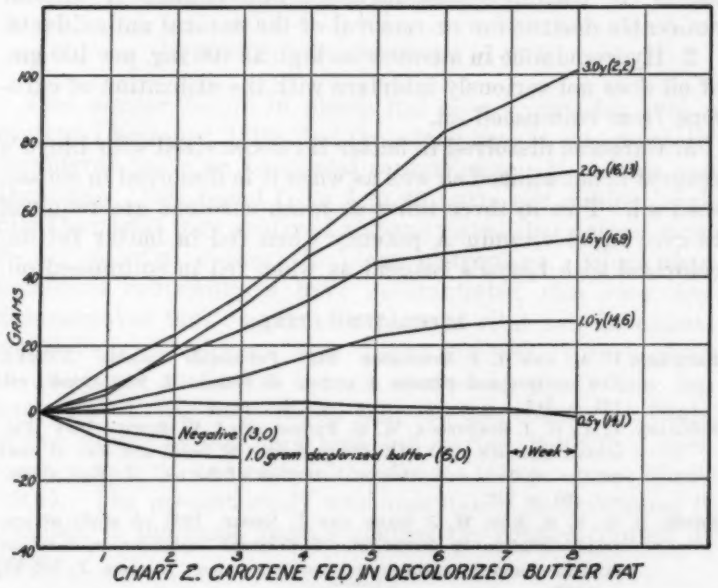

CHART 2. CAROTENE FED IN DECOLORIZED BUTTER FAT

Chart 2 Figures on curves indicate in micrograms the daily dose of carotene per rat. Figures in parentheses indicate the number of animals in the experiment and the survivors.

The high mortality of the animals that received carotene dissolved in decolorized butter fat even at relatively high levels of carotene intake, and which were making rapid gains suggests that the decolorization with Lloyd's reagent may have removed a factor which supplemented the vitamin A activity of carotene.

If this assumption were true it might account, in part at least, for the difference between the utilization of carotene from cottonseed oil and from the decolorized butter fat. This problem is now under investigation.

CONCLUSIONS

1. Treatment of melted butter fat with Lloyd's reagent removes the natural yellow pigments and vitamin A without noticeable destruction or removal of the natural antioxidants.

2. Hydroquinone in amounts as high as 100 mg. per 100 gm. of oil does not seriously interfere with the utilization of carotene from cottonseed oil.

3. Carotene dissolved in butter fat decolorized with Lloyd's reagent is not utilized as well as when it is dissolved in cottonseed oil. Two to three times as much carotene are required to give equal vitamin A potency when fed in butter fat decolorized with Lloyd's reagent as when fed in cottonseed oil.

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INFLUENCE OF SOIL AND VARIETY ON THE COPPER CONTENT OF GRAINS [1]

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That copper occurs in plants has been known for over a century (Meissner, 1816), but for a long time it was generally considered to be an accidental contaminant. Maquenne and Demoussy ('20) found from 3 to 40 parts per million in all plant material they analyzed and suggested that copper must be an essential element in plant metabolism. Since then numerous contributions have substantiated this view and demonstrated that copper also plays a vital role in animal metabolism (Elvehjem, '35). These discoverers have given to analytical studies of copper content of plants new importance. It has been shown that the ash content of Utah-grown grains is not only high but varies depending upon locality, variety, and cultural conditions (Greaves and Hirst, '29 a). The present study was undertaken to determine, if possible, if the same factors control the copper content of grains.

METHODS

Samples of grains and corresponding soil samples were collected from various localities throughout the state of Utah. These were prepared for analyses according to the methods of The Association of Official Agricultural Chemists ('30). The grains were analyzed for copper according to the modified Biazzo method of Elvehjem and Lindow ('29). After

NOTE: The varieties of wheat analyzed were furnished by Prof. A. F. Bracken and were grown on the Nephi Dry-farm Substation.

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considerable experimentation and careful checking which guaranteed reliable results, the following method was adopted for determining the copper content of the soil.

Thirty grams of soil were weighed into 500 cc. Erlenmeyer flasks, 60 cc. concentrated nitric acid and 5 cc. of concentrated hydrochloric acid being added and allowed to stand over night. It was then boiled for 30 minutes, diluted with water, filtered through a procelain filter, and washed to 400 cc. The filtrate was evaporated to dryness, from 3 to 5 cc. of concentrated hydrochloric acid added, and again evaporated to dryness. This process was repeated until practically all nitric acid was removed. Twenty cubic centimeters hydrochloric acid (1:1) were added, the solution warmed and washed into a 250 cc. flask with 125 cc. of hot water. The solution was then heated to boiling and hydrogen sulfide passed through until cold. The flask was stoppered, allowed to stand 1 hour, and tested for hydrogen sulfide saturation. The solution was filtered through paper pulp over a fine grade of filter paper and washed free of iron with approximately 100 cc. 0.3 normal hydrochloric acid saturated with hydrogen sulfide. The precipitate and filter paper were ashed and the residue taken up with 3 drops of nitric acid (1:9), 2 drops of hydrochloric acid (1:9), and 10 drops of concentrated sulfuric acid; this was heated to sulfuric acid fumes, the residue being made up to 25 cc. with distilled water and the copper determined in an aliquot by the modified Biazzo method of Elvehjem and Lindow ('29). The method, as outlined, was first checked on soils of known copper content and was found to recover over 98 per cent of the added copper. The water, chemicals, and apparatus were all checked for copper. All determinations were made in duplicate or triplicate and averages only are given.

RESULTS

Table 1 gives the parts per million of copper found in wheat and soil from various sections of the state.

Copper content of Utah wheats analyzed varied from 5.8 to 10.2 parts per million, with an average of 7.8. Webster

and Jansma ('29) reported the copper content of wheat from a number of states as varying from 4.2 to 8.7 parts per million, with an average of 6.0. This places Utah wheat slightly higher in copper content than for other states reported. Invariably the copper content of the soil was found to be higher than that of the wheat grown upon it. The copper content of the soil varied from 6.4 to 24.5, with an average of 16.7 parts per million. Hence, there was no concentration of the copper in the seed, as is usually the case with essential elements.

Deschamps, as early as 1848, suggested that a definite relationship existed between the copper content of plants and the copper content of the soil in which they were grown.

TABLE 1

Parts per million copper found in soil and wheat from different parts of Utah

SAMPLE LOCATION	COPPER CONTENT (P.P.M.)		SAMPLE LOCATION	COPPER CONTENT (P.P.M.)	
	Soil	Wheat		Soil	Wheat
Farmington	24.5	8.1	Howell	16.7	8.1
Fairview	22.7	8.7	Joseph	15.8	9.7
Fairview	22.7	10.2	Redmond	13.9	7.9
Cedar Valley	19.6	5.8	Hansel Valley	13.3	10.2
Utah County	18.9	6.3	Richfield	8.3	7.2
Utah County	17.5	6.3	Santa Clara	6.4	5.9
Cedar Valley	17.2	6.9			

Flinn and Inouye ('29), however, consider the copper content of plants to be due to a physiological factor and not related to the copper content of the soil.

Calculations, in regard to the coefficient of correlation between soil and wheat, were made according to the formula $r = \frac{\Sigma x y}{\sqrt{\Sigma x^2 \Sigma y^2}}$ which was 0.273, with a probable error of correlation 0.363. This indicates no relationship between copper content of the wheat and soil. Average results for copper determinations made on barley are given in table 2.

Samples of wheat, oats and barley were often taken from different localities, which accounts for the variation in the copper content of soil from the same farming district. The copper content of Utah barley analyzed varied from 6.2 to

11.9 parts per million, with an average of 7.8. Again, the soil was invariably higher in copper than was the grain and varied from 6.4 to 41.2 parts per million of copper with an average of 17.6. The coefficient of correlation of 0.136, with a probable error of 0.204, indicates no correlation between the copper content of soil and of grain.

Average results of copper determinations made on samples of oats are given in table 3.

TABLE 2

Parts per million copper found in soil and barley, Utah

SAMPLE LOCATION	COPPER CONTENT (P.P.M.)		SAMPLE LOCATION	COPPER CONTENT (P.P.M.)	
	Soil	Barley		Soil	Barley
Farmington	41.2	8.4	Joseph	13.5	9.8
Eden	27.4	6.5	Upper Hansel Valley	13.2	6.7
Fairview	22.7	6.5	Utah County	12.6	7.0
St. George	17.5	11.9	St. George	7.6	6.8
Redmond	14.3	8.0	Richfield	6.4	6.2

TABLE 3

Parts per million of copper found in soil and oats, Utah

SAMPLE LOCATION	COPPER CONTENT (P.P.M.)		SAMPLE LOCATION	COPPER CONTENT (P.P.M.)	
	Soil	Oats		Soil	Oats
Farmington	50.9	6.4	Utah County	13.1	8.5
Joseph	19.2	7.5	St. George	12.5	7.4
Eden	18.5	6.5	Santa Clara	4.1	6.8
Redmond	14.1	6.5	Richfield	3.9	9.8

The copper content of Utah oats analyzed varied from 6.4 to 9.8 parts per million, with an average of 7.4. Therefore, insofar as this survey goes, it tentatively may be concluded that the average copper content of wheat, oats and barley is practically the same. The soils on which oats were grown carried from 3.9 to 50.9 parts per million copper, with an average of 17.0. The coefficient of correlation is —0.478, with a probable error of 0.182. It was only in the case of the soils containing less than 6.4 parts per million of copper that a concentration of copper is found in the grain. Does

the copper requirement of grain approximate this figure? Sommer ('31), Lipman and Mackinney ('31) grew plants on synthetic media and found that they would neither mature nor produce seeds without copper. This they considered essential in all phases of plant growth; they found from $\frac{1}{16}$ to $\frac{1}{8}$ part per million sufficient for growth and production of seeds but considered that more was necessary for normal crop production.

Webster and Jansma ('29) compared the copper content of wheat from several states and found it to range from 4.2 to 8.7 parts per million of copper. According to these investigators there seems to be no relation between ash content and copper content of grain. Flinn and Inouye ('29) contend that the copper content of the plant bears no relation to the copper content of the soil. This is probably true where the copper content of the soil exceeds 6 parts per million. However, they postulate a relationship between the copper content of the plant and certain physiological properties. The protein and ash content of grains varies widely with the variety (Greaves and Hirst, '29 b). Will this also hold in the case of copper? In order to answer this question, sixteen varieties of wheat, grown on the same soil and under similar conditions, were analyzed for copper. Each yearly sample was composed of three replications; the samples used for analyses were further composited from wheat grown during each of 6 years. Each analyzed sample was a composite of eighteen samples. Results, as given in table 4, represent the averages of three or more closely agreeing determinations from such a composite sample.

There is a wide variation in the copper content of different varieties of wheat. Montana 36, although grown on the same soil and under similar conditions, had three times the copper content of Kofod x Turkey.

Wheat collected from various parts in the state varied from 5.8 to 10.2 parts per million of copper, with an average of 7.8. The sixteen varieties of wheat all grown under similar conditions showed variation in copper content from 5.6 to 16.7

parts per million, with an average of 9.7. Therefore, it would seem that variety is more important than soil, insofar as copper content of grains is concerned. However, where the copper content of the soil falls below a certain minimum, this may not necessarily hold. There are a number of cases on record where the copper content of plants has been increased by the use of copper fertilizers (Elvehjem and Hart, '29; Miller and Mitchell, '31). Lindow, Elvehjem and Peterson ('29) analyzed 158 foods and found none completely lacking in copper. Their figures range from 0.1 mg. of copper per kilogram of fresh celery to 44.1 mg. per kilogram of fresh

TABLE 4

Parts per million of copper found in different varieties of wheat, Utah, grown on same soil

VARIETY OF WHEAT	COPPER CONTENT (P.P.M.)	VARIETY OF WHEAT	COPPER CONTENT (P.P.M.)
Montana 36	16.7	Karamont	7.8
Kubanka	16.4	Black Hull	7.6
Turkey 926	13.9	Hard Federation	7.4
Tenmarq	12.3	Marquis	7.0
Kharkov Hayes 2	11.8	Kofod	6.5
Newturk	11.0	Alton	6.4
Regal	9.4	Chul	5.9
Early Baart	9.3	Kofod x Turkey	5.6

calf liver. Foods were classified in descending order of copper content, as follows: Nuts, dried legumes, cereals, dried fruits, poultry, fish, animal tissue, green legumes, roots and tubers, leafly vegetables, fresh fruits, and non-leafly vegetables.

Although there are districts in which the copper content of the soil is extremely low (Russell and Manns, '33) and the quantity occurring in the plants low enough to cause deficiency diseases in animals feeding solely upon them (McHargue, '25; Neal and Becker, '33; Sjollema, '33), yet the limited data presented in this paper point at least to the tentative conclusion that this is not to be expected in Utah.

SUMMARY

Wheat, oats and barley, together with the corresponding soils from different parts of the state, were analyzed for copper. The wheat varied from 5.6 to 16.7 parts per million copper, with an average of 8.8. The barley varied from 6.2 to 11.9 parts per million, with an average of 7.8. The oats varied from 6.4 to 9.8 parts per million, with an average of 7.4. Corresponding soils carried from 3.9 to 50.9 parts per million, with an average of 17.1 parts per million. The copper content of the grain was invariably lower than that of the soil until the copper content of the soil was below 6 parts per million. No correlation was found between the copper content of the grain and the soil on which it was grown.

Sixteen varieties of wheat grown on the same soil and under similar conditions varied in copper content from 5.6 to 16.7 parts per million, with an average of 9.7. It therefore appears probable that variety is the main factor in determining the copper content of Utah-grown wheats. It appears improbable from the limited data presented in this paper that copper is a limiting factor in plant or animal nutrition, insofar as Utah is concerned.

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THE VITAMIN A RESERVE OF EMBRYO AND BABY CHICKS

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TWO CHARTS

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It has been noted by Emmett and Peacock ('23), Beach ('24), Hart, Steenbock, Lepkovsky and Halpin ('24), Hauge, Carrick and Prange ('27), Holmes, Pigott and Menard ('30), Heywang and Titus ('32), Guilbert and Hinshaw ('34), Frohring and Wyeno ('34) and others that chick rations deficient in vitamin A produce unsatisfactory growth and in order to obtain optimum growth a chick ration should be rich in vitamin A. Hence those responsible for compounding chick growing rations ordinarily attempt to insure an adequate supply of vitamin A, by including in the ration a number of vitamin A rich products, with the hope that sufficient vitamin A will be present to meet all probable needs for growth. Obviously this procedure, which relies upon maintaining a large excess of vitamin A in chick growing rations, entails unnecessary waste. Unfortunately those who compound chick rations do not have definite information concerning the vitamin A requirements of growing chicks. Furthermore a review of the literature revealed little information concerning either the size of a baby chick's store of vitamin A or the amount of vitamin A that a normal chick requires for optimum growth. Accordingly a study has been

made of the vitamin A reserve of chicks just before, at, and subsequent to hatching.

For the purpose of this study 100 embryo and 298 young chicks of two breeds, Rhode Island Red (R.I.R.) and Barred Plymouth Rock (B.P.R.) were obtained from six sources. In determining the vitamin A reserve of embryo and young chicks attention was centered on the livers for Baumann, Riising and Steenbock ('34) have reported that 95 per cent of the total vitamin A store of rats is present in the liver and Sherman and Boynton ('25), Rosenheim and Webster ('27), Moore ('31), McCoord and Luce-Clausen ('34) and others have obtained similar results. Since the size of chick livers at the end of incubation did not permit of individual analyses the livers from ten chicks were pooled. The vitamin A content of the livers was determined by the Carr-Price ('26) antimony trichloride colorimetric method. As soon as the livers were removed they were ground, as suggested by Torrance ('33), with an equal weight of anhydrous sodium sulphate. The mixture was extracted 8 hours with anhydrous ether. The ether was volatilized and the resulting fat covered with an atmosphere of nitrogen. A weighed amount of the fat was dissolved in chloroform and the vitamin assay was made by the usual procedure.

The first determination of the vitamin A store of embryo chicks (R.I.R. and B.P.R.) was made with dead embryos (hatchery 'W') obtained at the end of the incubation period. Since it is a routine practice to remove all dead embryos from the incubator on the eighteenth day of incubation the embryos in question were 19 to 21 days old. Inasmuch as the unabsorbed yolk contains a store of vitamin A it was decided to determine the vitamin A content of both livers and unabsorbed yolks. Unfortunately it was impossible to dissect the unabsorbed yolks from the first three groups of embryos. The fat content of the livers from the six groups of dead embryos (table 1) varied from 11.5 per cent to 16.0 per cent. The average vitamin A content for the livers of the six groups varied from 8 to 14 blue units. The average fat content of

the unabsorbed yolks was 18.6 per cent. The blue units for three groups of yolks varied from 22 to 35 units per yolk.

Since exact information was not available concerning the time of death of the embryos it was decided to determine the vitamin A reserve of live embryos of known age. Accordingly embryos of two breeds (R.I.R. and B.P.R.) were obtained (hatchery 'S') when the embryos were 18 days old. The parent stock which produced the embryo chicks all received

TABLE 1

Vitamin A reserve of embryo chicks[1]

BREED AND SOURCE	AGE	WEIGHT OF CHICK	WEIGHT OF LIVER	LIVER FAT	BLUE UNITS PER LIVER	WEIGHT OF YOLK	YOLK FAT	BLUE UNITS PER YOLK	TOTAL BLUE UNITS OF LIVER AND YOLK
Dead embryo									
	days	*gm.*	*gm.*	*per cent*		*gm.*	*per cent*		
RIR-W	19–21	..	0.92	14.3	14	....	....	..	..
RIR-W	19–21	..	0.91	13.6	13	....	....	..	..
RIR-W	19–21	41	0.87	14.6	8	....	....	..	..
RIR-W	19–21	40	1.02	16.0	13	7.4	17.7	22	35
BPR-W	19–21	41	0.95	15.0	14	7.6	21.4	32	46
BPR-W	19–21	41	0.87	11.5	8	11.5	16.6	35	43
Average (3)		41	0.92	14.2	12	8.8	19.6	30	41
Live embryo									
RIR-S	18	..	0.47	10.7	9	15.0	27.0	87	96
RIR-S	18	..	0.55	13.8	6	13.5	26.4	76	82
BPR-S	18	..	0.47	10.9	8	14.9	27.1	79	87
BPR-S	18	..	0.58	14.7	6	13.6	26.2	76	82
Average		..	0.51	12.5	7	14.3	26.7	80	87

[1] Average values—groups of ten chicks.

the same ration and was maintained under the same environment. The livers from these embryos were about one-half as large as those of the older embryos and contained about 1½ per cent less of fat. The weight of the unabsorbed egg yolks, 14.3 gm., indicates that approximately one-fifth of the yolk had been absorbed by the eighteenth day of incubation. The results of the vitamin A assays of the livers and unabsorbed yolks of the four groups of 18-day embryo chicks show the livers to have an average value of 7 blue units and the unabsorbed yolks 80 blue units.

Attention was next given to the vitamin A store of chicks at the time of hatching. Three groups of baby chicks were removed from the incubator as soon as possible after hatching and killed when approximately 6 hours old. The average weight of the livers (table 2) from the thirty chicks was 0.89 gm. and they contained 17.2 per cent of fat. The average weight of the unabsorbed yolk was 8.3 gm. In other words about 55 per cent of the original yolk had been absorbed, indicating that 50 per cent more yolk was absorbed during the last 3 days than during the first 18 days of incubation.

TABLE 2

Vitamin A reserve of baby chicks[1]

BREED AND SOURCE	AGE	WEIGHT OF CHICK	WEIGHT OF LIVER	LIVER FAT	BLUE UNITS PER LIVER	WEIGHT OF YOLK	YOLK FAT	BLUE UNITS PER YOLK	TOTAL BLUE UNITS OF LIVER AND YOLK
	days	*gm.*	*gm.*	*per cent*		*gm.*	*per cent*		
RIR-S	0.25	44	0.88	17.9	10	8.8	25.7	57	67
RIR-W	0.25	44	0.94	16.4	17	8.1	22.2	68	85
BPR-S	0.25	43	0.86	17.2	8	7.9	24.6	50	58
Average		44	0.89	17.2	12	8.3	24.2	58	70
RIR-H	1	40	1.16	20.3	27	4.0	15.0	34	61
RIR-S	1	40	1.09	18.3	10	6.1	23.4	47	57
RIR-W	1	35	1.16	17.2	14	4.9	17.3	39	53
BPR-S	1	41	0.98	18.8	13	6.7	25.1	62	75
BPR-W	1	36	1.09	19.2	29	4.0	11.3	39	68
Average		38	1.10	18.8	19	5.1	18.4	44	63

[1] Average values—groups of ten chicks.

The vitamin A content of the livers and unabsorbed yolks of the 6-hour-old chicks from hatchery 'W' was materially larger than that of livers and yolks from hatchery 'S' chicks. According to the work of Sherwood and Fraps ('32) it is possible that this larger store of vitamin A may result from a difference in the vitamin A content of the rations of the breeding stock that produced the eggs for the two hatcheries. Bethke, Kennard and Sassaman ('27) and Ellis, Miller, Titus and Byerly ('33) found that the addition of cod liver oil to the ration of laying hens materially increased the vitamin A content of the eggs produced. It is also possible that the

vitamin A content of the eggs used by the two hatcheries may have been influenced by the rate of production of the breeding stock. Koenig, Kramer and Payne ('35) found 33 units of vitamin A per gram of yolk for low-producing birds as compared with 20 units for high-producing birds. The average vitamin A store of the 6-hour-old chicks was 12 blue units for the liver and 58 blue units for the unabsorbed yolk. Thus the combined liver and unabsorbed yolk store of these chicks was 70 blue units as compared with 80 blue units for 18-day-old embryos.

Five groups of chicks of two breeds obtained from three different hatcheries were killed 24 hours after hatching. The average weight of livers was 1.10 gm. The fat content of the livers was 18.8 per cent which is approximately 1.50 per cent higher than that of comparable 6-hour-old chicks. The weight of unabsorbed yolk, however, was found to be 5.1 gm. or approximately only two-thirds the weight of unabsorbed yolk of the chicks which were killed when 6 hours old. The fat content of the unabsorbed yolk had also decreased during the first day of life from 24.2 per cent to 18.4 per cent. The vitamin A assay of the livers and unabsorbed egg yolk of the 24-hour-old chicks gave an average value of 19 blue units for the livers and 44 blue units for the unabsorbed yolks. These data show a continued decrease of the baby chick's vitmain A reserve.

In order to determine the influence of the absorption of the egg yolk and the early utilization of food by baby chicks on their reserve of vitamin A, two lots of forty Rhode Island Red chicks were obtained from two different sources. The chicks from hatchery 'W' were obtained when approximately 6 hours old and divided into four groups of ten chicks each. One group was immediately killed and the remaining groups were killed, 1, 2 and 3 days later. The first three groups were not fed. The fourth group received mash and water at the end of the second day. The forty chicks from hatchery 'R' were not obtained until they were 2 days old. One group was killed at that time and the remaining groups were killed

1, 2 and 3 days later. The first two groups of chicks were not fed. After the third day mash and water was continuously available to the last two groups of chicks.

It will be noted (table 3) that the body weight of the chicks decreased until the chicks were fed. Thereafter their body weight increased. On the other hand the weight of the liver increased progressively with the age of the chicks. The weight of the unabsorbed yolk decreased from approximately 8 gm. at hatching to 0.6 gm. when the chicks were 5 days old. The vitamin A assay of the livers and the unabsorbed yolk showed a progressive increase in the vitamin A content of the liver

TABLE 3

Decrease in vitamin A following hatching[1]

BREED AND SOURCE	AGE	WEIGHT OF CHICK	WEIGHT OF LIVER	LIVER FAT	BLUE UNITS PER LIVER	WEIGHT OF YOLK	YOLK FAT	BLUE UNITS PER YOLK	TOTAL BLUE UNITS OF LIVER AND YOLK
	days	*gm.*	*gm.*	*per cent*		*gm.*	*per cent*		
RIR-W	0.25	44	0.94	16.4	17	8.1	22.2	68	85
RIR-W	1	40	1.12	19.8	17	5.0	22.8	50	67
RIR-W	2	38	1.18	19.8	19	3.8	15.5	33	52
RIR-W[2]	3	43	1.54	16.2	34	2.1	14.0	45	79
RIR-R	2	36	1.12	18.5	35	2.9	13.6	45	80
RIR-R	3	33	1.13	19.4	42	1.6	10.0	30	72
RIR-R[3]	4	44	1.57	14.1	36	0.9	13.0	40	76
RIR-R	5	48	1.82	11.4	59	0.6	13.9	22	81

[1] Average values—groups of ten chicks.

[2] Received feed at end of second day.

[3] Received feed at end of third day.

and a decrease in the vitamin A contained in the unabsorbed yolk.

In order to estimate the value of the unabsorbed yolk as a reserve source of vitamin A for utilization by the chick during the first 5 days of life, the data obtained from the assay of the unabsorbed yolk from the 198 chicks between the first and fifth day of life have been correlated. The average values obtained from these correlated data are reported as graphs (chart 1). Inspection of the data accumulated relative to the absorption of the egg yolk shows that 90 per cent of the yolk present at hatching is absorbed during the first 5 days of life. The fat content of the egg yolk

decreased even more rapidly for it dropped from 20.6 per cent in the unabsorbed yolk present in 1-day-old chicks to 13.9 per cent for the unabsorbed yolk present in 5-day-old chicks. The vitamin A units present in the unabsorbed yolk decreased from 49.5 units for the 1-day-old chicks to 22.3 units for the 5-day-old chicks. The combined vitamin A potencies of the liver and the unabsorbed yolk decreased from 65.5 units for the 1-day-old chicks to 51.6 units when the chicks were 3 days old. The chicks were fed at this time.

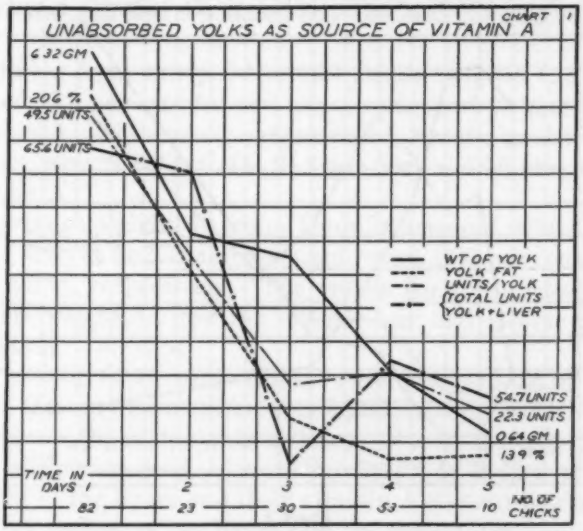

Chart 1

Subsequent to feeding the vitamin A potencies of the unabsorbed yolk and liver increased to 57.4 units when the chicks were 4 days old and then fell to 54.7 units when the chicks were 5 days old.

It is of interest to correlate the rate of growth of young chicks with their vitamin A reserve. Accordingly data in this connection have been assembled from studies of 298 chicks killed at different periods between the first and fourteenth day of age. The average values computed from these data

have been plotted as graphs (chart 2). The average body weight of the chicks at 1 day of age was 40.4 gm., but it decreased during the next 2 days, when feed is normally withheld from baby chicks, to 35 gm. Subsequently the body weight increased rapidly to 98.4 gm. when the chicks were 14 days of age. The average weight of the liver was 1 gm. when the chicks were 1 day old and it increased to 4 gm. when the chicks were 2 weeks old. The fat content of the

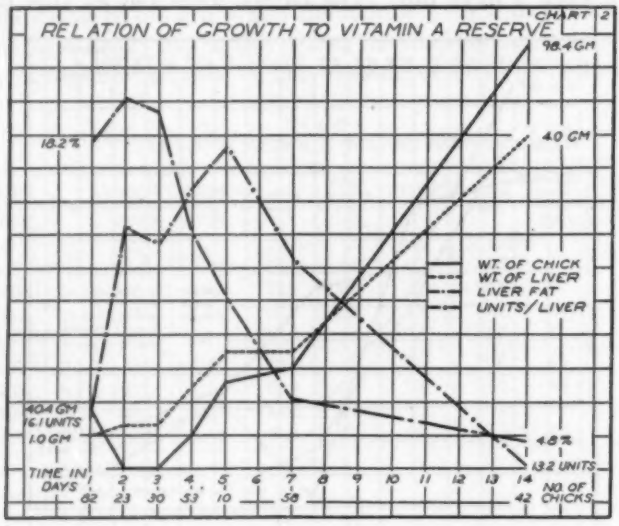

Chart 2

liver decreased from 18.2 per cent for 1-day-old chicks to 4.8 per cent for 14-day-old chicks. The vitamin A potency of the livers increased very rapidly from 16.1 units at 1 day of age to 32.2 units at 5 days of age, while the chicks were utilizing the vitamin A stores of the unabsorbed yolk. After the egg yolk had been absorbed, by the end of the fifth day of life, the vitamin A potency of the chicks' livers decreased rapidly to 13.2 blue units per liver when the chicks were 14 days old.

SUMMARY

Data concerning the vitamin A stores of embryo and young chicks have been accumulated by assaying the livers from 100 chick embryos and 298 young chicks. Since the unabsorbed egg yolk is a rich source of vitamin A these were assayed.

The livers from forty embryo chicks at the eighteenth day of incubation weighed 0.51 gm. and contained 12.5 per cent of fat and 7 blue units of vitamin A. The unabsorbed yolk weighed 14.3 gm. and contained 26.7 per cent fat and 80 blue units.

The livers from thirty 6-hour-old chicks weighed 0.89 gm. contained 17.2 per cent fat and had a reserve of 12 blue units of vitamin A. The corresponding values for the unabsorbed yolk were, weight 8.3 gm., fat 24.2 per cent and 58 blue units of vitamin A.

The average weight of livers from 24-hour-old chicks was 1.10 gm., the fat and vitamin contents were 18.8 per cent and 19 blue units, respectively. The values for the unabsorbed yolks were weight 5.1 gm., fat 18.4 per cent and 44 blue units of vitamin A.

Two lots of forty baby chicks were obtained from two sources. Groups of ten chicks were killed at 24-hour intervals. The body weight of the chicks decreased continuously until feeding was commenced. During the first 4 or 5 days of life the weight of the livers and their vitamin A content increased but the fat content of the livers and the weight, fat and vitamin A content of the unabsorbed yolks decreased. The rapid increase in the vitamin A content of the livers of chicks following hatching is doubtless influenced by the large store of vitamin A present in the egg yolks.

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THE AMINO ACID CONTENT OF EGGS AND CHICKS: RELATION TO DIET AND TO INCIDENCE OF CHONDRODYSTROPHY[1]

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Pollard and Carr ('24) and Gerber and Carr ('30) reported differences in the Van Slyke nitrogen distribution of eggs from pigeons on different diets. McFarlane, Fulmer and Jukes ('30) were unable to find any significant influence of diet on the amino acid content of hen eggs. Titus, Byerly, and Ellis ('33) reported evidence of a slight effect of the diet on the total nitrogen of the egg yolk. Calvery and Titus ('34) were unable to find any marked difference in the composition of the proteins of eggs from pullets on different protein diets.

Titus, Byerly and Ellis also found that certain protein diets tend to lower the hatchability of the eggs produced. Part of the chick mortality was shown to be caused by chondrodystrophy (Chondrodystrophia foetalis), a type of defective bone and cartilage formation of unknown etiology. Their evidence indicated an amino acid deficiency as a possible factor in the etiology of chondrodystrophy. Since this abnormality represents primarily a failure in cartilage development, we observed that gelatin, the best known protein in cartilage, contains about 22 per cent glycine. Corn, on the other hand, contains no glycine in its chief protein, zein.

[1] Paper no. 1382, journal series, Minnesota Agricultural Experiment Station. The data in this paper were submitted by A. Rae Patton to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of doctor of philosophy, June, 1935. All pertinent individual data are available for reference from the Division of Agricultural Biochemistry.

A corn ration was found by Byerly, Titus and Ellis ('33 a, '33 b) to permit an exceedingly high incidence of chondrodystrophy. It is for this reason that the glycine content of eggs was investigated with reference to the incidence of chondrodystrophy.

EXPERIMENTAL

The following rations were used:

Optimum ration	*Per cent*	*Deficient ration*	*Per cent*
Ground yellow corn	20	Ground yellow corn	70
Standard wheat middlings	20	Corn gluten (lot 39)	22
Ground oats	20	Cod liver oil	1
Bran	10	Wheat germ	3
Meat and bone meal	5	Oat hulls	1.5
Fish meal	5	Monosodium phosphate	0.9
Soybean meal	5	Calcium carbonate	1
Milk	5	Sodium chloride	0.5
Alfalfa leaf meal	6	NaCl, $CaCO_3$, corn	ad. lib.
Wheat germ	3		
Cod liver oil	1		
NaCl, $CaCO_3$, corn, wheat	ad. lib.		

Fourteen white Leghorn pullets were raised on the 'optimum' ration, and then restricted to the 'deficient' ration from May 4 to August 12, 1933. During this period average egg production dropped from eight eggs per day to less than two eggs per day. Eggs produced on the 'optimum' ration, and others produced near the close of the period on the 'deficient' ration were analyzed. The entire egg contents were extracted 48 hours each with acetone, alcohol, and ether, yielding samples containing 16.0 per cent nitrogen. In the case of chick samples,[2] the entire egg contents were treated in the same manner. A sample for analysis consisted of the contents of six eggs.

Tryptophane and tyrosine were determined by the method described by Folin and Marenzi ('29), histidine by the Rosedale-Da Silva method ('32), total surfur by the Denis method

[2] Chondrodystrophic material was identified and provided by Dr. F. B. Hutt, who also supplied the normal chicks for comparison.

('10). The results are given in table 1. Van Slyke nitrogen distribution was determined according to the Cavett modification ('32). The duplicate analyses are given in table 2. The glycine analyses shown in table 3 were made by the method described by Patton ('35), and the total creatinine by the method of Folin ('14). The glycine data for the egg

TABLE 1

Tryptophane, tyrosine, histidine and total sulfur determinations on eggs from hens fed optimum and deficient (corn) rations

	TRYPTOPHANE	TYROSINE	HISTIDINE	SULFUR
	per cent	*per cent*	*per cent*	*per cent*
Eggs from White Leghorns optimum ration	1.08 ± 0.06	4.27	2.26	1.3
Eggs from same flock after 14 weeks on corn ration	1.06 ± 0.05	4.28	2.35	1.4

TABLE 2

Van Slyke nitrogen distribution. The figures are expressed as percentage of total nitrogen

	EGGS FROM HENS FED				NORMAL CHICKS		CHONDRODYSTROPHIC CHICKS	
	Optimum ration		Deficient ration					
Ammonia	14.62	14.75	10.06	12.50	13.50	13.70	16.60	16.20
Humin	10.00	7.38	8.07	4.75	8.63	8.77	9.46	8.12
Filtrate amino	37.80	38.10	43.30	46.20	39.20	38.40	35.70	36.60
Filtrate non-amino	9.70	10.90	10.90	10.80	13.80	14.10	14.00	14.20
Arginine	9.75	9.75	11.00	11.00	8.77	8.77	8.77	7.80
Cystine	0.39	0.39	0.42	0.33	0.36	0.36	0.41	0.42
Histidine	6.40	6.40	3.90	4.55	6.90	7.60	5.40	6.70
Lysine	9.76	9.76	11.97	11.42	11.37	10.67	9.73	9.38
Total	98.42	97.43	99.62	101.55	102.53	102.37	100.07	99.42

samples represent the average of concordant results from single samples and for the chicks the mean of average data from two samples.

In addition to the samples described, eggs were collected from two white Leghorn pullets fed the optimum ration plus 1 gm. of glycine per day, in capsules. Glycine was determined in the eggs before and after 1 month of glycine feeding.

Glycine exerted toxic action in large doses (4 gm. a day). Two days of this treatment resulted in cessation of egg production. Food and water consumption were practically zero. Both hens exhibited extreme prostration, inability to stand, and sudden fits of agitation. When glycine feeding was discontinued, the symptoms rapidly disappeared, and normal egg production was resumed.

TABLE 9

Glycine and total creatinine determinations

	MEAN GLYCINE CONTENT OF TOTAL 'PROTEIN'	TOTAL CREATININE IN DRY MATTER
	per cent	*per cent*
Eggs from White Leghorns, optimum ration	2.21	
Eggs from same flock after 14 weeks on corn ration (glycine deficient)	2.16	
Eggs from hen R on optimum ration before feeding glycine	1.80	
Eggs from hen R on optimum ration plus glycine	1.87	
Eggs from hen L on optimum ration plus glycine	1.82	
Chick embryos which developed normally	9.5 9.1	0.02
Chick embryos which died from chondrodystrophy	6.8 6.3	0.02

DISCUSSION

On the basis of the glycine determinations certain conclusions may be drawn. In the first place, feeding a low glycine ration does not alter the glycine content of the eggs which the hen lays. Also, feeding a high glycine ration does not alter the glycine content of the eggs, either free or combined in protein. Since incubated eggs contain more glycine than fresh eggs, there appears to be a synthesis of glycine during embryonic development.

It is interesting that the total creatinine values found were the same in both normal and chondrodystrophic chick samples. This may constitute evidence of the similarity in age of the

chick groups compared, since total creatinine is known to increase rapidly during incubation (Needham, '31).

Any comparisons between the composition of the rations and of the chicks shown in table 2 would be purely theoretical, since the chondrodystrophic chicks were not produced on the 'deficient' ration used. The ammonia nitrogen values diverge in an unexpected fashion, as do the humin, filtrate amino, and lysine nitrogen values. The significance of this cannot be interpreted at the present time. Arrangements have been made for continuing the phase of this work concerning amino acids and chondrodystrophy.

SUMMARY

1. A significant difference was found between the glycine contents of normal and chondrodystrophic embryos.

2. A synthesis of glycine during development of the hen's egg was demonstrated.

3. Glycine was found to be toxic to hens when fed in large doses.

4. Feeding glycine to hens did not influence the glycine content of the eggs.

5. An investigation has been made of the amino acid content of eggs from fowls receiving 'optimum' and 'deficient' protein rations.

6. The analyses, including tryptophane, tyrosine, histidine, glycine, arginine, cystine, lysine and the Van Slyke nitrogen distribution, did not demonstrate a significant effect of the diet on the composition of the egg proteins.

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THE EXCRETION OF VITAMIN C IN NORMAL INDIVIDUALS FOLLOWING A COMPARABLE QUANTITATIVE ADMINISTRATION IN THE FORM OF ORANGE JUICE, CEVITAMIC ACID BY MOUTH AND CEVITAMIC ACID INTRAVENOUSLY

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FOUR FIGURES

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The application of the titration method to the estimation of vitamin C in urine has resulted in the recent suggestion that the urinary excretion of vitamin C can be used as a diagnostic test of vitamin C subnutrition in humans. Harris and Ray ('35) found that normal adults, on average diet, excreted from 15 to 30 mg. of vitamin C daily. The administration of large amounts of orange juice to such individuals resulted in a prompt and marked increase in the urinary output (Harris, Ray and Ward, '33; Hess and Benjamin, '34; Johnson and Zilva, '34). On the other hand, when the test dose was administered to patients with scurvy, or to those with a history of vitamin C underfeeding, the urinary excretion remained low. As a result of these observations it was suggested that the urinary excretion of ingested vitamin C was an index of the degree of saturation of the individual and therefore might be used as a test for hypovitaminosis C. Others have in general confirmed these observations but have reported a low urinary output after the test dose in some

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normal persons (Schröder, '35; Youmans, Corlette, Akeroyd and Frank, '35), in infectious diseases (Schröder, '35) and in some vascular disorders (Finkle, '35). Before such a test may be applied to clinical use, the range of physiologic variation in normal subjects, the effect of varying degrees of vitamin deprivation and the influence of various pathologic states on vitamin C excretion must be experimentally determined.

In this investigation the effect of variations in the intake of vitamin C on the urinary excretion has been studied under controlled conditions in a group of twelve normal adults. The 24-hour excretion was determined during a period of low vitamin C intake, and also during the daily administration of large amounts of vitamin C in the form of orange juice. After saturation, the effect of comparable amounts of vitamin C in the form of orange juice and of crystalline cevitamic acid ('Cebione' Merck & Co.) by mouth and of the latter intravenously was observed. In addition, the vitamin C content of whole blood was determined at appropriate intervals.

MATERIALS AND METHODS

Four female and eight male subjects were studied. Two of the women (L., J.C.) were graduate nurses, one (Je.C.) was a medical student and one (A.M.) a technician. Seven of the men were medical students, the other one a hospital interne. The age of the subjects varied between 21 and 30 years. With the exception of L.K., all were in excellent health and habitually ingested diets containing at least average amounts of antiscorbutic foods. L.K. gave a history of gingival bleeding for several months and of low dietary intake of vitamin C.

The first 24-hour urine sample was collected while the subject was taking his usual diet. During the following week the diet remained as before, except that all fresh fruits, vegetables, and tomatoes or tomato juice were eliminated. It was estimated that such restriction in the diet reduced the daily intake of vitamin C to a low level (10 to 20 mg.). This basal diet was continued throughout the remainder of the experiment except as noted below. During the second period of

8 days, 400 cc. of orange juice were given in a single dose each day after breakfast. During the third period the daily ration of orange juice was reduced to 200 cc. The orange juice was freshly squeezed from California oranges and contained 50 to 60 mg. of cevitamic acid per 100 cc., as determined by the titration method on a number of occasions. The value of 50 mg. per 100 cc. orange juice was used since the error in using this approximate value was no greater than that which is normally introduced in such dietary experiments. At the end of the third period 100 mg. of cevitamic acid were given first orally and then intravenously on successive days. Blood samples were taken for the determination of vitamin C content at the beginning of the first and second periods and at the end of the third period. At the conclusion of the experiment, four individuals took a vitamin C-free diet (meat, bread, cereals, pasteurized milk) for 2 days, and on the third day in addition received a large portion of orange juice (400 to 1000 cc.). Temperature and weight were recorded daily. No attempt was made to regulate activity; however, variations from the normal routine of any subject were recorded for reference.

Twenty-four-hour urine samples were collected from 7 A.M. to 7 A.M. daily. Each individual specimen was placed, as soon as voided, in a bottle containing glacial acetic acid to insure against loss of vitamin C. When not in use the bottle was kept in a cold, dark place. Incomplete 24-hour samples were discarded. Vitamin C content was determined by rapid titration of the urine against a measured volume of a standardized solution of 2:6-dichlorophenolindophenol, according to the method described in detail by Harris and Ray ('35). The requirements which Ahmah ('35) suggests are necessary for accuracy in the determination were met in these titrations. Determination of blood vitamin C were made in trichloroacetic acid filtrates of oxalated blood.

Capillary fragility determinations were made routinely each day by the method described by Dalldorf ('33).

RESULTS

During the initial period of curtailed vitamin C intake, several of the subjects complained of anorexia, fatigue and irritability. In the majority of instances these complaints were made voluntarily. Without exception, they disappeared within a few days after the administration of orange juice was begun. L.K. noted increased gingival bleeding during the period of vitamin C limitation but within a few days after taking large amounts of orange juice, bleeding from the gums entirely disappeared for the first time in several months. It is of interest that this subject, as indicated in table 2, had the lowest concentration of vitamin C in his blood in the beginning of the experiment and had the second highest value at the end of the high C diet. Inspection of the record of the urinary excretion shows the ease with which his tissues became saturated. Otherwise each subject remained in good health throughout the experimental period. No significant change was observed in the temperature or weight.

Estimations of the intake and 24-hour excretion of vitamin C in each of the twelve subjects are shown in figures 1, 2 and 3. Twenty-four-hour excretion while on a normal diet varied between 15 and 28 mg. The majority of the subjects continued to excrete vitamin C in approximately the same amount during the period of limited vitamin C intake. Two individuals (Je.C. and J.C.) excreted smaller amounts during this period.

Considerable variation was observed in the response to the addition of orange juice. G.A. and J.C. showed significant increases in urinary output on the first day. R.A., on the other hand, continued to excrete only small amounts of cevitamic acid until the ninth day, when 45 per cent of the test dose appeared in the urine. In the other subjects the urinary content of vitamin C began to rise on the second, third or fourth day after the addition of orange juice. Fluctuations also occurred after saturation had presumably been accomplished. A few individuals (G.A., A.M. and L.K. in particular) consistently excreted large amounts of the test dose each

day. Others showed striking and unexplained variations. In several subjects (G.A., L., A.M., J.F. and L.T.) the 24-hour excretion exceeded 100 per cent the daily test dose within a few days after the administration of orange juice was begun. The same test dose on subsequent days, however, resulted in

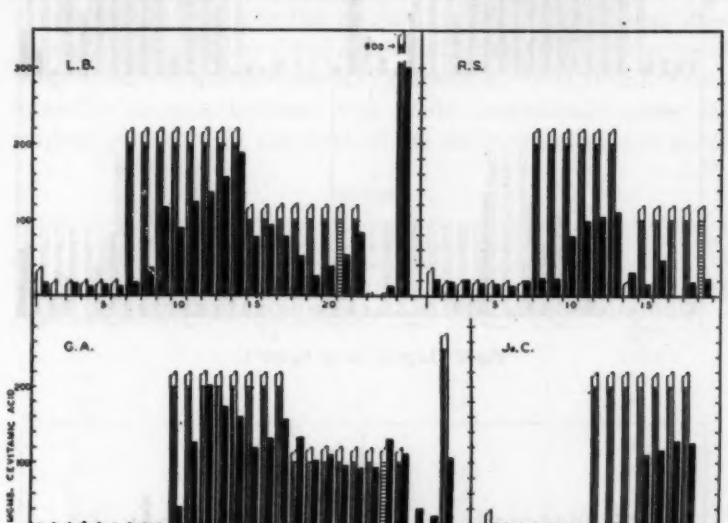

Fig. 1 The daily intake and 24-hour excretion of cevitamic acid. Open columns indicate estimations of vitamin C in the diet. Columns with vertical lines indicate vitamin C administered as orange juice. Columns with horizontal lines indicate vitamin C given as cevitamic acid by mouth, cross-hatched columns, cevitamic acid intravenously. The 24-hour excretion of vitamin C in the urine is represented by the solid columns. Blank spaces for the latter indicate that the 24-hour specimen was incomplete and therefore discarded.

the elimination of smaller amounts, which, in several instances, were but little above the control level. Reduction in the size of the test dose resulted in a roughly comparable decrease in excretion.

In table 1 are summarized the results of the administration of orange juice and of cevitamic acid by mouth and intravenously. Although here again, considerable individual variation

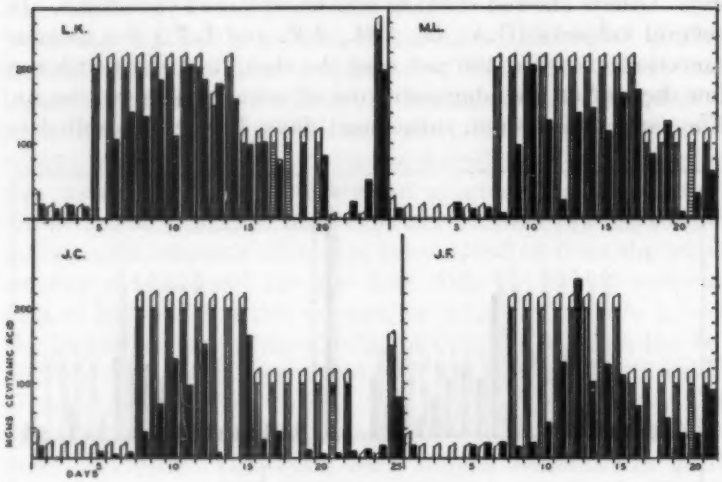

Fig. 2 Legend as in figure 1.

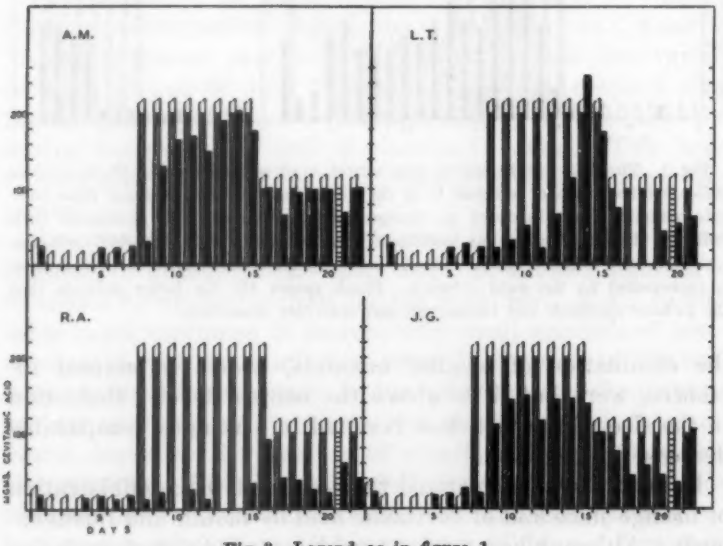

Fig. 3 Legend as in figure 1.

was observed, the averages show that approximately the same proportion of the vitamin is excreted when given in equivalent amounts as orange juice and as crystalline cevitamic acid. The urinary output is almost doubled, however, by intravenous administration.

In three individuals the hourly excretion of the test dose was determined before and after saturation. The results of these observations are shown in figure 4. The initial determination in each instance was made immediately after the control period, with the first of the daily doses of 400 cc. of

TABLE 1

Twenty-four-hour excretion of cevitamic acid in response to test doses of vitamin C after saturation with orange juice

SUBJECT	200 CC. ORANGE JUICE (APPROXIMATELY 100 MG. CEVITAMIC ACID)	100 MG. CEVITAMIC ACID BY MOUTH	100 MG. CEVITAMIC ACID INTRAVENOUSLY
	mg.	*mg.*	*mg.*
G.A.	93.4	129.2	110.9
R.A.	19.6	60.4	40.2
L.B.	51.0	52.0	82.7
J.C.	6.4	5.7	9.6
F.	33.0	...	91.5
J.G.	44.1	34.0	105.00
L.	8.8	34.5	63.8
A.M.	98.0	69.5	102.0
S.	18.2	22.6	....
L.T.	43.5	54.3	63.8
Average	41.6	41.3	73.3

orange juice, representing approximately 200 mg. of vitamin C. Subsequent determinations after saturation were made following the administration of 100 mg. of vitamin C given, usually on successive days, as orange juice and as crystalline cevitamic acid by mouth and intravenously. When orange juice and cevitamic acid were taken by mouth, the urinary content of vitamin C began to increase during the first hour and reached a peak in from 3 to 6 hours. After intravenous administration, however, a large proportion of the test dose was excreted in the first hour. Significant differences in the excretion curves before and after saturation

were apparent despite the fact that the initial dose was twice the size of those employed later. As might be expected, the introduction of the material directly into the blood stream resulted in its excretion in the urine more quickly and more completely than when taken by mouth.

The results of withdrawal of vitamin C in four individuals after saturation are included in figure 1. The urinary excretion of three subjects dropped promptly to the control level;

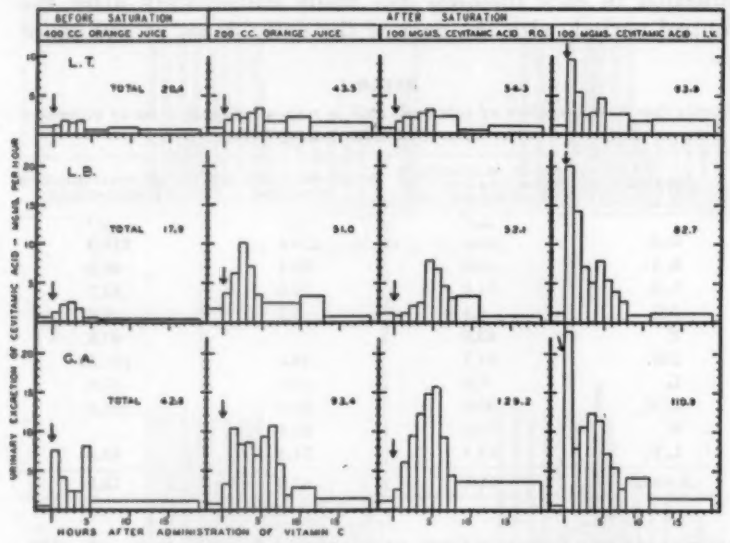

Fig. 4 The hourly excretion of test doses of vitamin C before and after saturation with orange juice. Arrow indicates time of vitamin C administration.

the fourth excreted amounts somewhat above those observed during the control period. In each instance, the administration of a large amount of orange juice after 2 days of strict vitamin C deprivation resulted in the excretion of a large proportion of the test dose.

Determinations of the vitamin C content of whole blood are shown in table 2. Although in several cases higher values were obtained after saturation, the differences are small and

probably without significance. These figures are, on the whole, slightly lower than those recently reported in a large series of individuals by Mirsky, Swadesh and Soskin ('35) who likewise observed no correlation between the ascorbic (cevitamic) acid content of blood and the dietary regime.

No significant changes were noted in capillary fragility. Detailed results of this phase of the experiment will be reported in a later paper.

TABLE 2

Whole blood content of vitamin C

SUBJECT	BEGINNING OF PERIOD 1 (NORMAL DIET)	BEGINNING OF PERIOD 2 (AFTER LOW-C DIET)	END OF PERIOD 3 (AFTER HIGH-C DIET)
	mg. per cent	*mg. per cent*	*mg. per cent*
G.A.	0.69	0.81	1.21
A.	1.32	1.00	1.15
L.B.	1.00	0.93	1.63
Je.C.	1.02	0.88	...
J.C.	1.19	1.72	1.25
F.	0.93	0.83	1.00
J.G.	0.95	1.19	0.86
L.K.	0.58	1.16	1.47
L.	0.92	0.96	1.40
A.M.	0.94	0.83	0.85
R.S.	1.07	0.91	...
L.T.	0.98	0.80	1.10
Average	0.96±0.037	1.00±0.047	1.21±0.051

Difference 1 and 2 = 0.04±0.063.
Difference 1 and 3 = 0.25±0.064.
Difference 2 and 3 = 0.21±0.066.

DISCUSSION

In several respects the observations here reported confirm those previously recorded by other investigators. The values for the urinary excretion of vitamin C by normal individuals on an average diet compare favorably with those reported by Harris and Ray ('35) in England, by Schröder ('35) in Germany and by Youmans, Corlette, Akeroyd and Frank ('35) in this country. We are also able to confirm the observation (Harris and Ray, '35) that in the majority of individuals the normal amounts of vitamin C continue to be

excreted for at least several days after the dietary intake of antiscorbutic foods has been curtailed. A delay of from 1 to several days in the excretion of a daily test dose of vitamin C was observed in individuals whose tissues had presumably become 'unsaturated' by a short period of vitamin deprivation. This observation apparently supports the contention that the reserve stores of such individuals are quickly depleted and must be repleted before the ingested vitamin C appears in more than normal amounts in the urine.

It is noteworthy that considerable variation was observed in the time required for increased urinary excretion to begin after the addition of orange juice to the diet. Such variation might be expected if the control period were of insufficient duration to reduce the subject to a common level of unsaturation. On the other hand, it is possible that individuals differ in the efficiency with which vitamin C is absorbed, stored and excreted.

After saturation had presumably been accomplished, wide fluctuations were observed in the urinary response to repeated daily test doses.

These observations suggest that factors other than the 'state of saturation' of an individual may influence the urinary excretion of vitamin C after a given test dose of orange juice.

Further studies may reveal hitherto unrecognized pitfalls in the method used in determining the vitamin C content of urine, or factors which have not been satisfactorily controlled in these experiments; a larger group of normals might show a decreased percentage of variability. Unless the variations in the response of apparently normal individuals can be predicted or controlled, they offer a serious limitation to the clinical use of the test suggested for the diagnosis of subclinical vitamin C deficiency states.

SUMMARY

1. The effect of variations in the daily intake of vitamin C on the urinary excretion of cevitamic acid has been studied in twelve normal young adults.

2. The urinary excretion of vitamin C by individuals on an average normal diet varied between 15 and 28 mg. per 24 hours.

3. Excretion continued at a steady rate during a preliminary control period of low vitamin C intake.

4. Considerable individual variation was observed in the urinary response to repeated test doses of orange juice, both during and after apparent saturation with vitamin C.

5. Comparable amounts of vitamin C given orally as orange juice and as cevitamic acid resulted in similar urinary excretion curves. Cevitamic acid administered intravenously was excreted more rapidly and more completely than when given by mouth.

6. Variations in the intake of vitamin C had no demonstrable effect on the cevitamic acid content of whole blood, or on the capillary fragility.

The authors wish to express their appreciation to Dr. S. W. Clausen for his interest and helpful suggestions.

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THE ROLE OF CALCIUM AND PHOSPHORUS IN DETERMINING REPRODUCTIVE SUCCESS

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SEVEN TEXT FIGURES AND ONE PLATE

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Many factors influence successful gestation and lactation: one of these is the calcium and phosphorus content of the diet. Deficiencies of these elements are not as immediately apparent as are deficiencies in dietary organic constituents (Meigs, '22), but usually appear only after several reproductive cycles. Simmonds ('24) observed that on a diet deficient in calcium (0.103 per cent) rats were able successfully to suckle as many as two litters of young but that they subsequently failed even to reproduce.

Most reports in this field have embraced but a short span of the life cycle of the experimental animal: the period of growth, or fractions thereof; isolated observations throughout gestation; or, the period of lactation. In view of the relative incompleteness of short periods of observation we thought it desirable to study the behavior of experimental animals receiving constant amounts of dietary calcium and phosphorus throughout the entire span of their reproductive life. We hoped that such life-time observations would enable us to draw definite conclusions regarding the optimal calcium-phosphorus relationship in pregnancy and lactation.

LITERATURE

The observation quoted above (Simmonds, '24) is the only report we have found regarding the specific type of problem in which we were interested, although Hunscher ('30) studied

calcium balances for two successive lactation periods in women. Sherman and Muhlfeld ('22) have studied reproductive behavior on certain milk diets, but their diets involved changes in many variables.

Many investigators have reported optimal Ca:P ratios, although Sherman ('32 a) warns against such statements without exhaustive research, his thought being that the optimal ratio will change throughout the different life stages, and from species to species. Usually such ratios have been based on growth curves (Bethke, Kick and Wilder, '32), on blood serum values (Kramer and Howland, '32), or on bone-ash determinations (Brown, Shohl, Chapman, Rose and Saurwein, '32) in the rat, although calcium balances in the lactating cow (Ellenberger, Newlander and Jones, '32), and goat (Hart, Steenbock, Kletzien and Scott, '26–'27) have been widely employed. Optimal ratios for the chick (Wilgus, '31) have been determined by measuring growth and bone ash. The effect of different ratios on the strength of bone of growing pigs was studied by Bethke, Edington and Kick ('33). Such investigations have in general indicated Ca:P ratios between 1.0 and 2.0 as optimal. In recent years the relation of calcium and phosphorus to rickets has received much attention (Bethke, Kick and Wilder, '32; Kramer and Howland, '32; Brown, Shohl, Chapman, Rose and Saurwein, '32).

Based upon the mineral retentions of infants and children, Stearns ('31) considers a Ca:P retention of less than 1.5:1.0 indicative of rapid muscular or tissue growth, and a retention ratio of 2.0 indicative that the majority of retained mineral elements are used to form bone. In early infancy she finds the ratio of retention is less than 1.5, but that in childhood it approaches 2.0.

During lactation, retention of calcium and phosphorus is admittedly poor. Metabolism studies with the lactating cow and nursing woman (Donelson, Nims, Hunscher and Macy, '31) emphasize the strain on maternal mineral reserves during this period. The first part of lactation was usually characterized by negative calcium balances, and only when milk flow diminished was calcium stored. Additional vitamin D

during this first period may (Hart, Steenbock, Kletzien and Scott, '26–'27), or may not (Hart, Steenbock, Teut and Humphrey, '29) change a negative to a positive balance, although it usually improves the balance. The literature on this subject has been reviewed by Macy, Hunscher, McCosh and Nims ('30).

Negative calcium balances are commonly observed during gestation. Toverud and Toverud ('31) obtained forty-four balances on sixteen expectant mothers receiving the ordinary institutional diet, occasionally supplemented. The following table is compiled from their findings.

Calcium and phosphorus balances at different levels of intake compiled from the data of Toverud and Toverud ('31)

LEVEL OF INTAKE		AVERAGE INTAKE	NUMBER METABOLISM PERIODS	AVERAGE MONTH OF PREGNANCY	NEGATIVE BALANCES, PER CENT
Calcium	Less than 1 gm.	0.83	17	7.6	41.2
	More than 1 gm.	1.35	27	7.6	14.8
Phosphorus	Less than 1 gm.	0.82	6	7.0	83.3
	More than 1 gm.	1.48	38	7.7	13.2

Their results show, fairly definitely, that when the mineral intake is above 1.0 gm. daily, positive balances may be expected, but that intakes below this level predispose to negative balances. They feel that the level of mineral intake is a more important factor in obtaining positive balances than the presence of vitamin D. On the other hand, Macy, Hunscher, Nims and McCosh ('30), frequently obtained negative balances with calcium intakes considerably greater than 1.0 gm. The adult maintenance allowance of Sherman ('32 b) (0.68 gm.) is evidently insufficient for periods of mineral stress. The fact that frequently retention during pregnancy does not meet the mineral requirement of the fetus has been emphasized (Coons and Blunt, '30).

The foregoing incomplete survey may be summarized from a nutritional standpoint as follows:

1. Optimal Ca:P ratios, for various species, have been provisionally established at 1.0 to 2.0, although Sherman's ratio is 0.5 ('32 b).

2. Gestation is frequently accompanied by negative calcium balances. Where positive balances are obtained, retention is frequently less than calculated fetal requirement.

3. The level of mineral and vitamin D intake are of importance.

4. Early lactation is accompanied by negative mineral balances in spite of adequate intake. Vitamin D may be of value during this period. Late lactation, when milk flow has declined, is the period of storage.

5. The only evidence adduced for a change in optimal ratios of intake during different life periods is from retention data. As yet, no important species difference has been reported.

6. Continued low calcium intake may result in reproductive failure.

EXPERIMENTAL

One hundred and forty-five virgin rats, carefully selected from our stock colony,[1] were mated and allowed to raise their first litter while receiving stock food (Bills' modification of the Steenbock diet).[2] The technic employed with this control litter was routinized, and employed for ten subsequent reproductive cycles on experimental diets, litters were reduced to six pups at birth, the young were killed and ashed at 21 days of age, and the mother was remated on the day of weaning.

After successfully raising their first litters on stock food, five mothers were placed on each experimental diet, and their average performance during ten subsequent cycles taken as an expression of the suitability of a given calcium-phosphorus level and ratio for gestation and lactation. In order to evaluate performance three criteria were chosen: 1) the average weight of the young at 21 days; 2) their percentage ash; and 3) the change in weight of the mother throughout

[1] Rats weighing 190 to 230 gm. (average 199.4), at 79 to 127 days (average 99) were selected. They were considered unsuitable if 1) they did not become pregnant after one mating; 2) if they gave birth to less than six young; 3) if they did not raise young to 21 days of age; 4) if the young weighed less than an average 25 gm. at 21 days; and 5) if the mother failed to gain weight.

[2] Yellow maize, 57.0; whole milk powder, 25.0; linseed oil meal, 12.0; crude casein, 3.7; alfalfa leaf meal, 1.5; iodized table salt, 0.4; calcium carbonate, 0.4.

the cycle. For the purpose of grading relative performance ten mothers were carried through the entire eleven cycles on stock food.

Basal diet. The basal diet consisted of:

Casein (acid washed)	20.0
Dextrinized starch	50.1–55.1
Lard (Swift's Silverleaf)	9.0
Yeast concentrate (equivalent in B to 24 per cent, in G to 8 per cent, yeast)	4.0
Wheat germ oil	1.6
Carotene solution in oil 3: 1000	0.3
Salts—Ca and P free[1]	3.1
Rice cellulose	1.2– 5.9

This diet contained 0.018 per cent calcium and 0.245 per cent phosphorus. No vitamin D was added, and the animals were housed in a room protected from ultraviolet light. Two-kilogram batches of each diet were mixed not less often than every 2 weeks, and, when not in use, stored at 10°C. Record was made of individual food and water consumption.

Calcium-phosphorus mixtures. To the basal diet predetermined quantities of dicalcium phosphate and either monobasic ammonium phosphate or calcium acetate, were added. The maximum amount of dicalcium phosphate was used, and the additional required element made up by one of the other two salts. The dietary levels of the two elements are indicated in the following table, together with the diet number and Ca : P ratio.

TABLE 1

Levels and ratios of calcium and phosphorus employed

PERCENTAGE PHOSPHORUS IN DIET	PERCENTAGE CALCIUM IN DIET									
	0.245		0.490		0.735		1.225		2.450	
	Diet No.	Ca/P Ratio	Diet No.	Ca/P Ratio	Diet No.	Ca/P Ratio	Diet No.	Ca/P Ratio	Diet No.	Ca/P Ratio
0.245	1	1.0	6	2.0	11	3.0	16	5.0	21	10.0
0.490	2	0.5	7	1.0	12	1.5	17	2.5	22	5.0
0.735	3	0.33	8	0.66	13	1.0	18	1.66	23	3.33
1.225	4	0.2	9	0.4	14	0.6	19	1.0	24	2.0
2.450	5	0.1	10	0.2	15	0.3	20	0.5	25	1.0

[1] Composition: NaCl 24.50, $MgSO_4$ 9.14, $KHCO_3$ 59.20, KCl 3.84, Fe citrate 2.94, $CuSO_4$ 0.32, $MnSO_4$ 0.04, KI 0.02 per cent.

For completeness, two additional diets were included: Diet no. 26, (the basal ration unsupplemented), and diet no. 27, containing 0.122 per cent calcium and 0.245 per cent phosphorus. (Results with these two diets are reported in tables 2 and 6, and figure 4).

Controls. Two groups of five rats each were used as controls (stock diet). One group was kept in the stock breeding room under conditions that prevail for our rat colony (sterilized wood shavings for bedding and tap water in the drinking bottles). The other group was kept in the experimental room on wire screens—given distilled water to drink, and stock food from our standard iron-plate cup. The laboratory was kept at constant temperature and humidity.

Evaluation of results. For convenience in presenting the data, we have expressed our findings on experimental diets in terms of their percentage relation to the performance of the controls.

1. The weight of young at 21 days of age was converted to a percentage figure as follows:

$$\frac{\text{Average weight experimental young at 21 days}^{4}}{\text{Average weight stock control young in corresponding gestation}} \times 100 \qquad (1)$$

2. Numerous analyses of stock young, 21 days of age, gave an average ash content of 2.775 per cent[5] (Cox and Imboden, '36). It was our original thought that this figure would remain fairly constant for the stock controls, and be independent of the cycle number. By definition 2.775 per cent ash was perfect performance (100 per cent). The lowest percentage body ash we were able to produce after 24 days on McCollum's rachitic diet no. 3143 was 2.000. Regarding this as zero, the

[4] In arriving at the average weight, the number of young that should have been raised (the number born, or a maximum of six) was employed. This procedure was likewise used in computing the average weight of the controls, i.e., 10 × 6 rats should have been raised, during each cycle, by the controls.

[5] Made on the basis of gross dead weight. Before ashing, the gastro-intestinal tract was removed. The carcasses were placed in weighed silica dishes and ashed in an automatically controlled muffle at 550°C.

ash content of the experimental young was converted to a percentage figure as follows.

$$\frac{0.775 - (+ 2.775 - x)}{0.775} \times 100 \qquad (2)$$

when x is the observed percentage body ash. When x is greater than 2.775 all indicated signs are reversed.

3. The net change in the weights of the mothers during the cycle was converted to a percentage basis, as follows:

$$100 - ([+ x - y] \times \tfrac{1}{3})^{*} \qquad (3)$$

when x is the average change in weight of the controls in the particular cycle, and y is the change in weight of the experimental mothers. When y is greater than x all indicated signs are reversed.

The sum of the three individual grades of the five mothers in each group, divided by fifteen gave the net grade for the cycle. The average of ten cycle grades gave the 'success' rating of the diet. When cessation of reproductive activity, or death, occurred, grades of zero were given in subsequent cycles.

RESULTS

We present in table 2 the composite results obtained in this investigation. The compilation permits comparison of any two diets but is not suitable for analysis of the experiment as a whole. Diet no. 13 has been omitted because of the obvious unsuitability of the mothers.

Final success rating. In figure 1 we have plotted the average success rating for the ten cycles (three factors evaluated) against the Ca/P ratio of the diet. There are five curves, indicating five levels of calcium intake. The ratings of the controls are indicated by 'X.'[7]

* '⅓' was included in this formula through the following argument: a 300 gm. rat can lose approximately 66 per cent of its weight before death. If the maximum possible loss (200 gm.) is assumed zero, and the net average gain of the controls assumed 100, 1 gm. gain or loss can be evaluated as ⅓ per cent.

[7] The grades are less than 100 because of death of the mothers, and a lowered percentage ash of their young.

TABLE 2

The effect of different amounts of dietary calcium and phosphorus on reproduction and lactation

DIET				GESTATION					LACTATION			FACTORS EVALUATED			FINAL 'SUCCESS' RATING	AVERAGE DURATION EXPERIMENT, DAYS	CLINICAL TUMORS	BONE ASH OF MOTHERS	FOOD AND WATER INTAKE			
																			Gestation		Lactation	
																					Increase per pup	
Diet no.	Per cent Ca	Per cent P	Ca/P ratio	Per cent fertile matings	Number completed	Number incomplete	Total living young	Number young available to raise	Number completed	Number young raised	Per cent available young raised	Average weight young at 21 days	Ash content grade	Change in weight of mother					Calories per rat per day	Water cubic centimeters per day	Calories per day	Water cubic centimeters per day
1	0.245	0.245	1.0	90.2	36	1	292	201	35	190	94.5	42.23	90.4	+46	76.0	435	2	61.88	63.9	29.4	8.5	3.9
2	0.245	0.490	0.5	84.9	42	3	302	220	33	156	70.9	43.32	75.7	+36	79.9	540	2	63.58	66.7	31.5	8.7	3.9
3	0.245	0.735	0.33	89.6	43	0	271	222	41	192	86.5	41.42	77.9	+86	93.6	536	4	62.92	62.3	29.4	7.0	3.7
4	0.245	1.225	0.2	80.4	38	3	293	213	36	188	88.3	41.79	84.6	+57	83.5	542	1†	65.98	59.9	40.1	7.7	5.0
5	0.245	2.450	0.1	65.0	12	1	90	68	2	8	11.8	16.60	142.7	—34	10.3	197	0	64.84	53.0	65.9	4.7	...
6	0.490	0.245	2.0	89.6	43	0	330	230	40	206	89.6	43.30	72.1	+55	90.4	527	1	64.56	65.1	33.3	9.7	3.9
7	0.490	0.490	1.0	76.8	42	1	314	229	38	188	82.1	46.66	93.7	+71	95.8	497	2	65.18	59.5	31.8	9.9	3.2
8	0.490	0.735	0.66	79.2	38	4	278	186	28	112	60.2	43.68	86.0	+84	67.3	472	3	66.12	63.5	30.3	8.6	2.9
9	0.490	1.225	0.4	83.7	36	0	315	206	31	146	70.9	41.29	89.4	+74	69.9	481	2	63.92	63.9	34.7	7.5	4.6
10	0.490	2.450	0.2	71.4	23	2	192	131	19	88	67.2	22.54	120.7	—92	35.2	341	0	62.99	57.7	58.6	6.6	3.6
11	0.735	0.245	3.0	72.9	42	1	292	209	36	163	77.5	32.34	69.6	+50	72.8	522	2	64.68	61.5	33.0	8.3	2.6
12	0.735	0.490	1.5	84.6	44	0	319	237	35	179	75.5	44.43	105.7	+58	93.9	493	2	65.82	66.7	36.1	8.5	2.8
14	0.735	1.225	0.6	83.0	38	1	299	214	37	182	85.0	42.68	93.0	+55	83.8	472	1	63.67	59.5	34.2	8.9	5.0
15	0.735	2.450	0.3	78.6	22	0	164	119	15	55	46.2	24.14	85.8	—83	26.8	333	1	61.28	54.0	59.9	4.5	2.6
16	1.225	0.245	5.0	70.6	12	0	94	68	10	51	75.0	21.39	59.2	—32	13.2	381	0	61.83	62.7	30.3	5.1	2.7
17	1.225	0.490	2.5	86.7	38	1	311	211	36	188	89.1	33.74	75.3	+53	73.1	471	1	63.70	58.7	32.4	7.2	3.8
18	1.225	0.735	1.66	90.9	39	1	294	216	35	159	73.6	45.35	98.1	+61	83.4	450	2	65.35	56.3	27.6	10.2	3.4
19	1.225	1.225	1.0	82.4	40	2	318	217	29	141	65.0	42.52	107.1	+105	77.8	431	1†	65.61	61.9	28.6	8.5	3.0
20	1.225	2.450	0.5	76.9	30	0	242	166	24	104	62.7	32.50	102.2	—50	46.4	350	0	57.08	55.8	54.6	6.5	4.2
21	2.450	0.245	10.0	....	2	0	19	12	0	0	0.0	....	....	—126	0.5	185	0	59.40	40.5	22.6	....	...
22	2.450	0.490	5.0	....	2	0	20	12	0	0	0.0	....	....	—40	0.8	351	0	61.36	34.4	21.1	....	...
23	2.450	0.735	3.33	62.1	18	0	124	95	12	57	60.0	19.16	85.7	—42	20.7	362	0	62.60	58.4	40.3	7.7	3.0
24	2.450	1.225	2.0	62.5	21	4	185	119	21	110	92.4	37.33	99.2	+31	40.2	365	1	65.22	61.4	30.2	8.8	3.4
25	2.450	2.450	1.0	90.0	35	1	295	193	19	82	42.5	33.51	105.1	+22	52.8	383	0	63.23	62.5	39.4	6.9	3.4
26	0.017	0.245	0.07	50.0	13	4	99	69	5	22	31.9	18.87	92.7	+5	15.0	261	0	57.77	53.1	28.3	5.3	...
27	0.122	0.245	0.5	68.4	25	1	197	137	17	80	58.4	31.59	92.9	+7	38.1	359	3	61.51	61.1	30.2	7.6	2.7
Stock[1]	0.51	0.47	1.1	79.6	39	0	357	231	38	213	92.2	48.20	92.5	+64	86.1	507	1	63.80	71.6	....	12.2	...
Stock[2]	0.51	0.47	1.1	87.5	42	0	338	234	39	185	79.1	48.73	81.5	+50	91.9	430	0	64.46	66.1	31.9	11.0	3.2

[1] In colony breeding room.
[2] In experimental room.

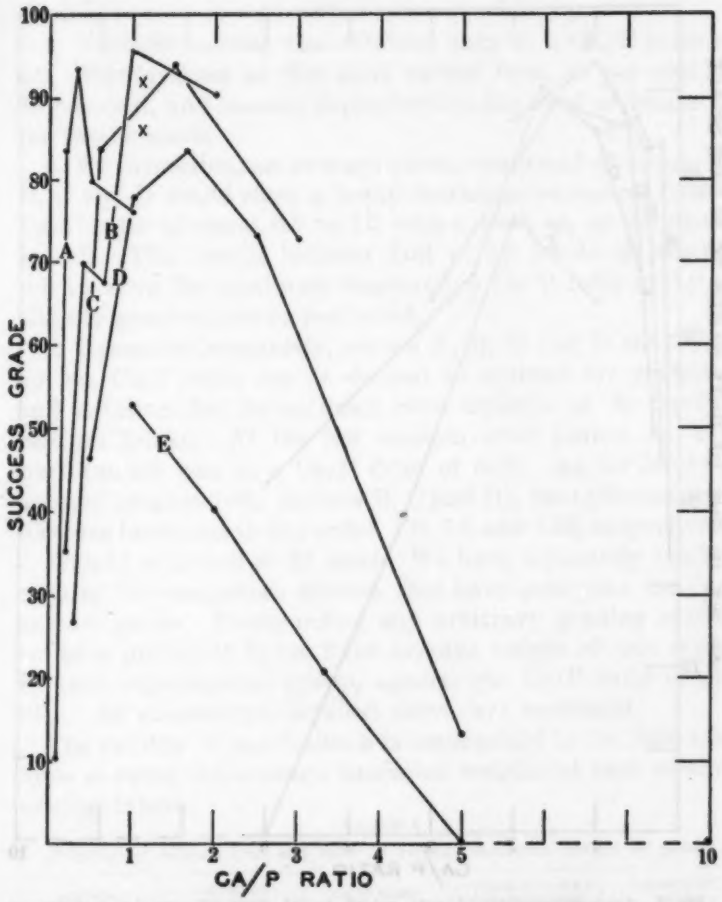

Fig. 1 The 'success' grade of groups of mother rats receiving various levels and ratios of calcium and phosphorus, against the Ca/P ratio of the diet. Levels of calcium are indiated by letters: Curve A, 0.245 per cent level; B, 0.490 per cent; C, 0.735 per cent; D, 1.225 per cent; and E, 2.45 per cent. The grade of the controls is indicated by 'X.'

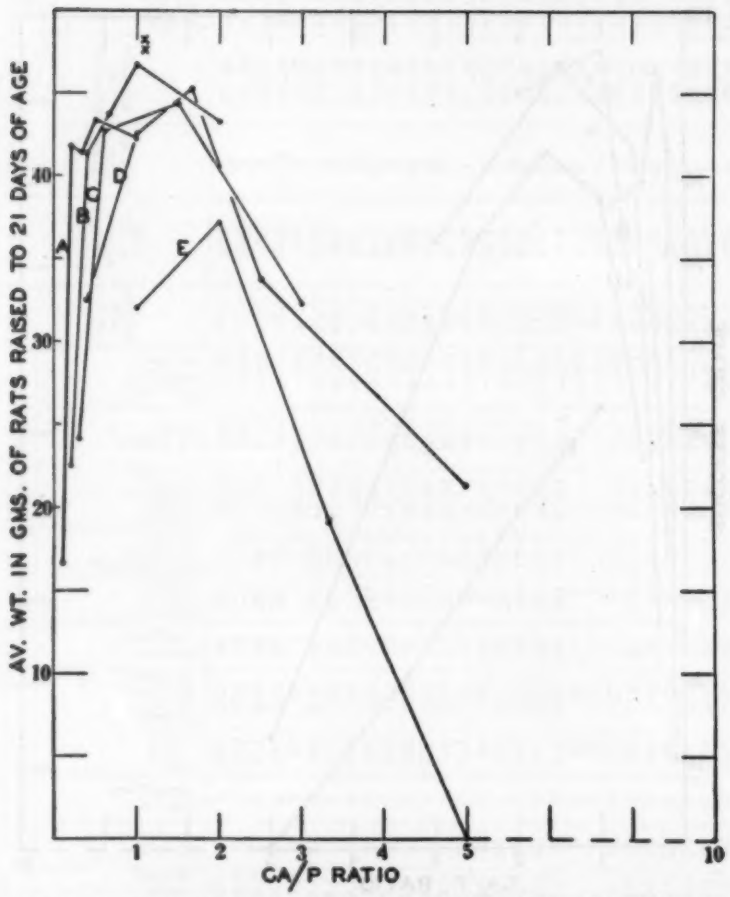

Fig. 2 Average weights of rats raised to 21 days of age by mothers on various experimental diets, against the Ca/P ratio of the diet. Calcium levels are indicated by letters: Curve A is the 0.245 per cent level; B, 0.490 per cent; C, 0.735 per cent; D, 1.225 per cent; and E, 2.45 per cent. The weight of the young from the control mothers is indicated by 'X.'

An examination of the curves warrants the following conclusions:

1. Variable success was obtained even at a Ca/P ratio of 1.0. Performance at this ratio varied from 53 per cent to 96 per cent, and seemed dependent on the *level* of intake of the two elements.

2. By inspection, an average curve, composed of curves A, B, C and D would show a broad maximum extending from a Ca/P ratio of about 0.5 to 1.5 with a peak at, or very near to, 1.0. This would indicate that at all levels of calcium intake, save the maximum employed, a Ca/P ratio of 1.0 or slightly greater is to be preferred.

3. Examined separately, curves A, B, C and D show that *no one Ca/P ratio* can be defined as optimal for gestation and lactation, for the optimum ratio depends on the *level* of calcium intake. At the low calcium level (curve A) best performance was at a Ca/P ratio of 0.33. As the level increased progressively (curves B, C and D), the optimum ratio likewise increased in the order, 1.0, 1.5 and 1.66, respectively.

Weight of young at 21 days. We have separately studied each of the component criteria that have gone into the final success grade. Disregarding any arbitrary grading system we have plotted in figure 2 the average weight of rats raised by each experimental group, against the Ca/P ratio of the diet. All conclusions, detailed above, are confirmed.

The validity of conclusion 3 is emphasized in the following table showing the average maximum weights at each level of calcium intake:

TABLE 3

Relation of calcium level and ratio to average maximum weight of young

DIET NO.	Ca LEVEL PER CENT	Ca/P RATIO	AVERAGE MAXIMUM WEIGHT OF RATS	NUMBER OF RATS
2	0.245	0.50	43.32	156
7	0.490	1.00	46.66	188
12	0.735	1.50	44.43	179
18	1.225	1.66	45.35	159
24	2.450	2.00	37.33	110

The actual differences in average weight between the maximum value and that adjacent to it on any curve is frequently small. We have determined the statistical significance of such differences, and the results are compiled in table 4. Only one significance ratio is less than 3.0 (2.8) so that the differences, though small, are significant, and the conclusion based on them, valid.

TABLE 4

The significance of the maximum weights of the five curves of figure 2

CURVE	CALCIUM LEVEL, PER CENT	(1) MAXIMUM		(2) RIGHT OF MAXIMUM		(3) LEFT OF MAXIMUM		SIGNIFICANCE RATIO [2]	
		Number of observations	P.E.[1] of average weight	Number of observations	P.E.[1] of average weight	Number of observations	P.E.[1] of average weight	(1)–(2)	(1)–(3)
A	0.245	156	0.34	190	0.21	192	0.27	2.8	4.4
B	0.490	188	0.30	206	0.21	112	0.33	9.3	6.7
C	0.735	179	0.34	163	0.26	182	0.25	28.3	4.0
D	1.225	159	0.38	188	0.23	141	0.25	26.3	6.4
E	2.450	110	0.38	57	0.37	82	0.54	34.1	5.7

[1] Probable error. Formula $P.E._m = 0.8453 \times$ the average deviation, divided by the square root of the number of observations.

[2] Calculated by dividing the square root of the sum of the squares of the probable error of the two means into the difference between the means. When the significance ratio is 3.0 or greater the difference is considered statistically significant.

Body ash of young at 21 days. In figure 3 we have plotted the grades obtained by formula (2) against the Ca/P ratio of the diet.

Disregarding the exceptionally high values at very low Ca/P ratios it is evident that the highest grades at all levels of calcium intake were obtained at a ratio of 1.0. This is somewhat surprising in view of the former finding that the optimum ratio for weight was dependent on the level of calcium intake; but it is not inconsistent with this finding. Obviously, maximum weight may not be paralleled by maximum ash.

Thinking in terms of 'grade' magnifies the numerical differences of percentage ash.[3] Thus, curve A shows grades of

[3] To calculate percentage body ash from the grade, multiply the latter by 0.00775, and add 2.000.

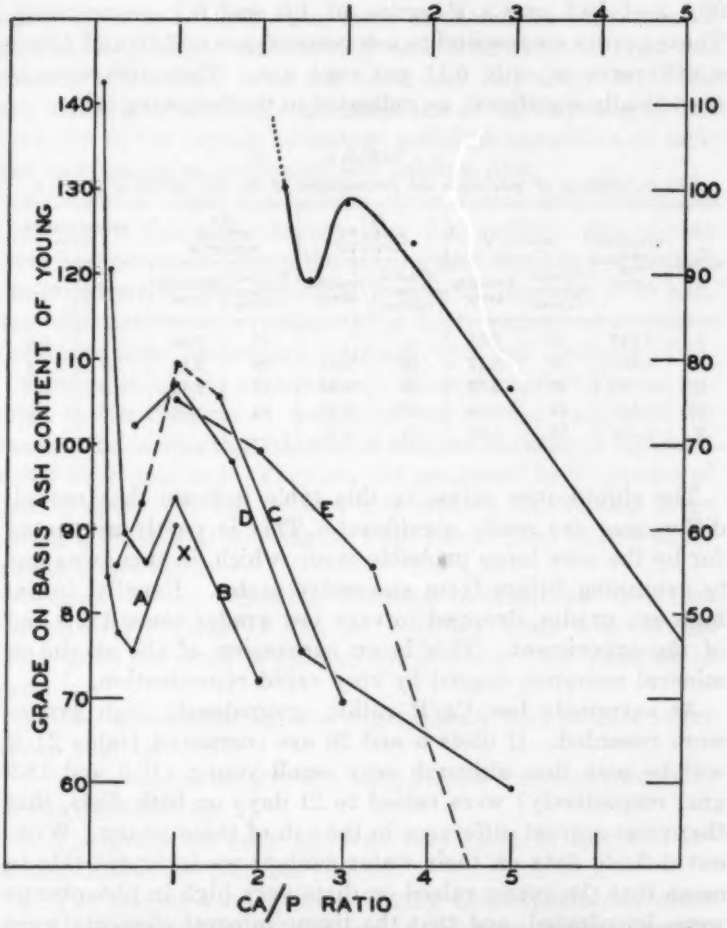

Fig. 3 Total ash contents of 21-day-old young raised by mothers on various experimental diets, against the Ca/P ratio of the diet (formula 2). Calcium levels are indicated by letters: Curve A, 0.245 per cent level; B, 0.490 per cent; C, 0.735 per cent; D, 1.225 per cent; and E, 2.45 per cent. Diet no. 13 was included. The ash content of young from the controls is indicated by 'X.' By moving the coordinates an average curve was constructed in the upper right of the figure.

90.5 and 75.5 at Ca/P ratios of 1.0 and 0.5, respectively. These grades correspond to ash percentages of 2.70 and 2.59—a difference of only 0.11 per cent ash. This difference is statistically significant, as indicated in the following table.

TABLE 5

The significance of maximum ash percentages of the five curves of figure 3

CURVE	CALCIUM LEVEL, PER CENT	(1) MAXIMUM		(2) RIGHT OF MAXIMUM		(3) LEFT OF MAXIMUM		SIGNIFICANCE RATIO	
		Number of observations	Probable error	Number of observations	Probable error	Number of observations	Probable error	(1)–(2)	(1)–(3)
A	0.245	35	2.51	..	...	33	2.40	..	4.2
B	0.490	38	2.17	40	2.03	28	3.81	7.3	1.8
C	0.735	17	3.53	35	3.28	37	2.12	0.8	4.0
D	1.225	29	3.04	35	3.33	24	3.34	2.0	1.1
E	2.450	19	3.37	21	4.35	...	..	1.1	..

The significance ratios in this table indicate that not all differences are really significant. This is partly accounted for by the very large probable error, which, in turn, was due to averaging litters from successive cycles. Usually, initial high ash grades, dropped to very low grades toward the end of the experiment. This is an expression of the strain on mineral resources caused by very rapid reproduction.

At extremely low Ca/P ratios, anomalously high grades were recorded. If diets 5 and 26 are compared (table 2) it will be seen that although very small young (16.6 and 18.9 gm., respectively) were raised to 21 days on both diets, that there was a great difference in the ash of these young. Without definite data on their water content we interpret this to mean that the young raised on diets very high in phosphorus were dehydrated, and that the tissue mineral elements were sufficiently concentrated to account for the high ash content. This thought is further confirmed by our studies on the bone ash of these young, which are reported separately (Cox and Imboden, '36). In spite of a high total ash content, their per cent bone ash was low. On such diets, therefore, total ash content is no indication of bone calcification. Diet no. 26,

however, with a Ca/P ratio even more severe than that of diet no. 5 produced rats equally small, but with normal ash content and normal bone ash (Cox and Imboden, '36). We are inclined to attribute the small size of these young to the inability of the mother to lactate sufficient quantities of milk and calcium on an extremely low calcium diet.

Excessively high ash grades were found only when the Ca/P ratio was more severe than 0.3, and the phosphorus level 2.45 per cent. Thus, the rats on diet 20 (2.45 per cent P, Ca/P ratio 0.5) did not show a greatly lowered bone ash, and did not have, presumably, a high phosphorus rickets (compare their percentage bone ash, Cox and Imboden, '36).

Change in weight of mothers. When formula (3) was applied to the changes in weight of the mother rats, and the results (table 2) graphed, curves similar to those of figures 1 and 2 were obtained. They are not presented here because of lack of space. It was evident that mother rats raising large young gained well in weight, while those that raised small young lost weight.

Because of the following itemized findings, we feel that the mineral content of diet no. 7 (Ca/P ratio 1.0, calcium level, 0.490 per cent) is optimal for gestation and lactation in rats:

1. The success rating was higher than that of any other group (fig. 1).
2. The average weight of young raised was exceeded only by the controls (fig. 2).
3. The grade on body ash was very close to 100, and exceeded that of the controls (fig. 3).
4. The mother rats gained, during the experiment, more than the controls. Diet 7 was one of five groups exceeding the controls in this respect (table 2).

The effect of raising dietary calcium. When diets of low, but constant, phosphorus content are compared, the effect of raising the calcium level is apparent (fig. 4). Presented graphically for successive cycles, the success rating seems regulated by the calcium level. At a very low level (curves

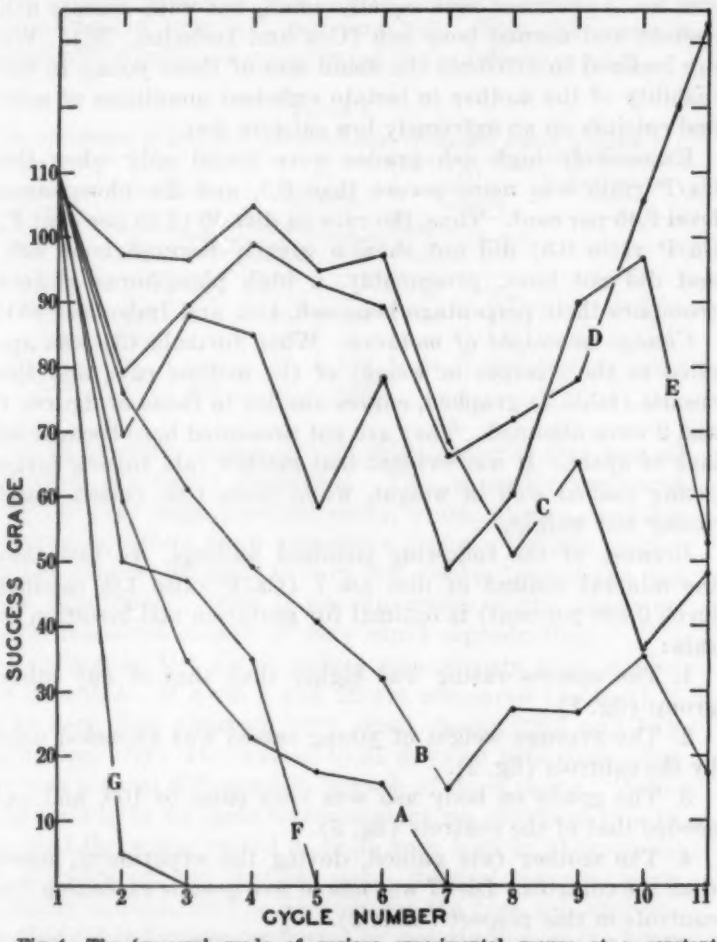

Fig. 4 The 'success' grade of various experimental groups at a constant phosphorus level (0.245 per cent), but various levels of calcium, plotted for successive cycles. Calcium level of curve A is 0.017 per cent; B, 0.122 per cent; C, 0.245 per cent; D, 0.490 per cent; E, 0.735 per cent; F, 1.225 per cent, and G, 2.45 per cent.

A and B) progressive decline in reproductive success was the prominent feature. At higher levels (curves C and D) excellent performance was obtained, and even at a Ca/P ratio of 3.0 (curve E) no great deleterious effect was noted. However, at still higher calcium levels (curves F and G) rachitic diets were indicated by progressively poorer performance.

The point we wish to emphasize is that although success on a given diet may be measured as relatively good for a single cycle, the true adequacy or inadequacy of the dietary intake can only be determined subsequently. An examination of curves B and D at cycle no. 2 (first experimental cycle) and a comparison of these first grades with later ones, indicate this fact. Similarly, curve C shows excellent performance (equalling that of the controls) for six cycles, and only in the last four cycles does the mineral inadequacy of the diet become apparent. Comparison with curve D indicates how performance can be consistently sustained even in the later cycles.

The success ratings of these diets are presented in table 6.

TABLE 6

Effect of increasing calcium intake

CURVE LETTERED	PER CENT Ca	PER CENT P	DIET NO.	AVERAGE SUCCESS RATING
A	0.017	0.245	26	15.0
B	0.122	0.245	27	38.1
C	0.245	0.245	1	76.0
D	0.490	0.245	6	90.4
E	0.735	0.245	11	72.8
F	1.225	0.245	16	13.2
G	2.450	0.245	21	0.5

Excess calcium vs. excess phosphorus. In the plan of our experiment there were equal numbers of groups having Ca/P and P/Ca ratios greater than 1.0. We therefore had an opportunity to determine which element is better tolerated in excess, calcium or phosphorus. Disregarding the mineral level, we have plotted in figure 5 the average weights of young raised against the Ca/P or P/Ca ratio of the diet. The position of the diet, on either side of the center ratio (1.0)

depended upon which element was in excess. Diets with identical ratios were averaged so that the relatively low position of the point at Ca/P ratio 1.0 is due to the low average weight from diet no. 25.

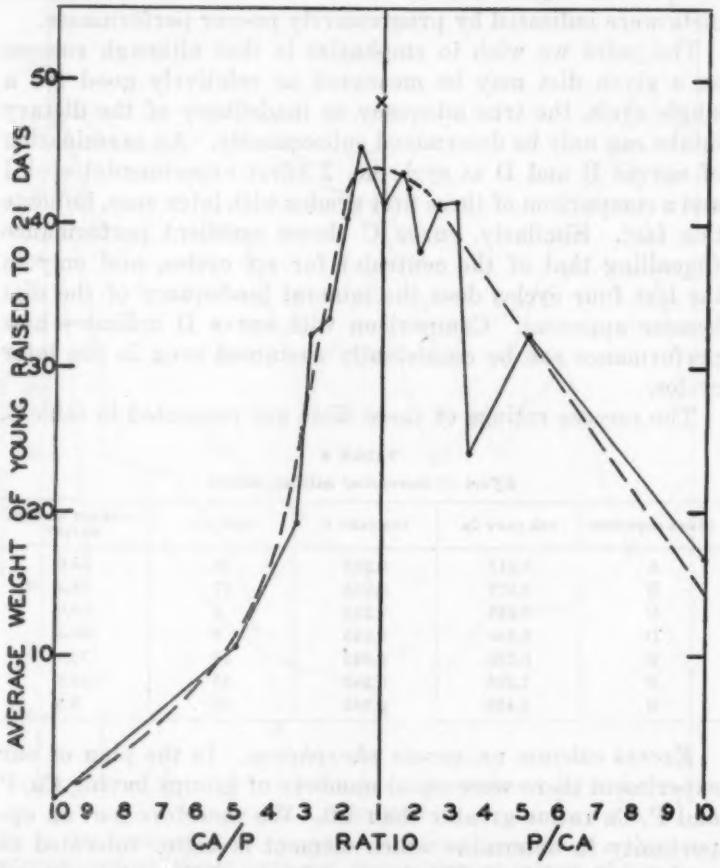

Fig. 5 The average weight of young raised to 21 days by mothers on different experimental diets, plotted against the Ca/P or P/Ca ratio of the diet. The position of the diet on either side of the central line (ratio 1.0) was determined by which element (P or Ca) was in excess. An equal number of experimental diets are recorded on either side of the central line. Groups with identical ratios were averaged. The grade of the controls is indicated by 'X.'

It is immediately evident that on the high calcium side of 1.0, the curve is very steep, and ascends rapidly, while on the high phosphorus side, the curve starts higher (indicating young of greater weight) and the ascent is much more gradual.

Averaging the weights for all diets on the high calcium side (omitting all with Ca/P ratios of 1.0), we obtained a net average weight of 27.7 gm.; while a similar average on the high phosphorus side gave an average of 35.0 gm. The conclusion is obvious; i.e., that phosphorus in excess is better tolerated by rats, than is calcium.

Bone ash of mothers. At the end of the experiment all mothers were killed, and the bone ash of the right femur determined in the usual way. The percentage bone ash (table 2) gave but little indication of the true condition of the bone. In general, it may be said, that diets high in phosphorus (nos. 5, 10, 15, etc.) gave bones extremely thickened, and soft. The bones were easily broken on removal, and frequently had multiple fractures which had healed with large, deforming lumps of bony (but not hard) material. We would term the bones fibrotic, were it not for the fact that the mineral matter was not reduced. Such bones were likewise found with diet no. 25, even though the calcium-phosphorus intake was balanced. Rachitic diets did not give bones especially low in ash, and the greatest reduction was observed in those diets most deficient in calcium, nos. 26 and 27.

Tumors. It has been shown, clinically (Babcock, '35), that lactation does not increase the incidence of mammary tumors. Our experimental animals, however, were afflicted to a surprising degree with such growths. As this is in contradistinction to the incidence in our stock colony, where prolonged rest periods between cycles are allowed, we were inclined to think that the large incidence was related to the rapid sequence of lactation required in this work. More recent observations, however, have made us believe that age is possibly an important factor. McCay, Crowell and Maynard ('35) have recently reported a tumor incidence of 16 per cent in stock animals. An attempt was made to remove surgically all tumors as soon as they were noted. Dr. W. C. Caldwell

examined histologically three typical examples (diet nos. 3, 9 and 12) and found them benign fibro-adenomas of the breast. Histologically, he noted considerable calcium deposits.

In table 2 the number of rats in each group showing tumors is recorded. Obviously there was no dietary distribution.

Calculi. Recent experimental work (Higgins, '33) has confirmed earlier observations (Osborne and Mendel, '17) that prolonged deficiency of vitamin A may cause bladder and kidney stones. Obviously, other factors are also involved (Keyser, '35). Our rats received adequate amounts of vitamin A (as carotene) but on certain diets, high in calcium, stones were observed; viz., diet 11, one rat; diet 18, one rat; diet 22, one rat; diet 23, two rats. On the latter diet (see

TABLE 7

Analysis of renal calculi

STONE FROM RAT NUMBER	PER CENT ASH	IN CALCULUS			IN ASH		
		Per cent Ca	Per cent P	Per cent Mg	Per cent Ca	Per cent P	Per cent Mg
85	52.54	34.69	0.78	1.22	66.03	1.48	2.32
115	52.29	34.60	0.86	1.39	66.17	1.65	2.65

plate 1, actual size), rat no. 115 had five bladder stones weighing 2.955 gm. (dried), and rat no. 114 had the entire renal calyx literally packed with calcium deposits. Rat no. 85 (diet 18) had three bladder stones, and much 'sand.' Plate 1 shows the relatively enormous size of these deposits. The largest stone measured $1.47 \times 1.02 \times 0.80$ cm. Analysis showed the stones to consist almost entirely of calcium salts with only traces of phosphate (table 7).

Food and water intake. For six experimental cycles record was made of food and distilled water intake. The average figures are given in table 2. Caloric consumption during gestation was, to some extent, dependent on the mineral level and ratio of the diet. A low calcium level (diet nos. 26 and 27) did not result in increased food consumption. High calcium or phosphorus levels at unfavorable ratios (diet nos. 5, 10, 15, 21 and 22) resulted in decreased food consumption. During lactation the caloric consumption increased to ap-

proximately the same extent on all diets (calculated per pup) save those most unbalanced in mineral elements. The increase due to lactation was greatest in the stock controls. Water intake was roughly equivalent in all groups, save those high in phosphorus. On such diets (4, 5, 10, 15 and 20) abnormal quantities were ingested. On the other hand, diets high in calcium did not cause such increases. In partial explanation, phosphorus is largely excreted in the urine; calcium is unabsorbed, or normally excreted through the large bowel, and therefore does not require additional fluid for its elimination.

DISCUSSION

Our results in this investigation have led us to the view that the optimum Ca/P ratio for gestation and lactation in rats is dependent upon the calcium level, the optimum ratio increasing as the calcium increases. This finding was based upon a study of our 'success ratings,' and the average weight of young raised by the different groups of mothers. On the other hand, a consideration of the body ash of the weaned young indicated that at all calcium levels a Ca/P ratio of 1.0 gave the greatest percentage ash, although all differences could not be regarded as statistically significant.

When our data was rearranged and examined at constant phosphorus levels, clear cut results were not obtained. We employed two criteria, success ratings and weights of young. The findings are presented in table 8.

When we examined the statistical significance of the average weights at a given phosphorus level, it was found that, at some levels, the average weight at the optimum ratio was not significantly different from the average weight at some other ratio. This is indicated in the last column of table 8.

We feel that the results are not sufficiently straight-forward to permit generalization. For example, it might be maintained that at all levels of phosphorus a P/Ca ratio of 1.0 was optimal; and with perhaps equal certitude, that the optimal P/Ca ratio increased with the phosphorus level until phosphorus constituted 0.735 per cent of the diet.

During the course of this work we were surprised that many of our rats, receiving a synthetic diet, performed as well as, or better than, the controls. This is in contradistinction to other reports on synthetic diets (Hartwell, '33). We are inclined to credit such results to an adequacy of the B vitamins. These were included at a high level because of the increased requirement during lactation (Sure, '27).

The decline in ash content of the young (with successive litters) does not appear when the average ash contents are reported. Such a decline was true in the control litters as well as in the experimental groups, although we had hoped that the ash percentage of the former would remain relatively constant throughout successive cycles. Sherman and MacLeod

TABLE 8

Relation of phosphorus level and optimum P/Ca ratio (compare table 3)

PHOSPHORUS LEVEL PER CENT OF DIET	OPTIMUM P/Ca RATIO		
	Basis: Success rating	Basis: Average weight young	Possible optimum ratio: see text
0.245	0.5	0.5	1.0
0.490	1.0	1.0	...
0.735	3.0	0.6	...[1]
1.225	1.66	1.66	1.0
2.450	1.0	1.0	...

[1] Diet 13, ratio 1.0, was not included. See page 153.

('25) have stated that the calcium content of virgin females is higher than that of males, but that after they had raised young, their calcium content resembled that of males. The decline in percentage ash of the young noted in this work was accompanied by an increase in the average weight, so that the actual weight of ash in their bodies increased (fig. 6). The fact that young from older mothers are larger, has been noted before (Abt, '23). Graphic record of the behavior of our stock control groups is given in figure 7.

Because the lactating animal is able, physiologically, to make adjustments between dietary intakes of calcium and phosphorus, and that supplied to the nursing young in the milk, the wide differences that have been noted in the growth of weaned young when placed on such severe ratios as we have employed, were not observed in this work. At a Ca/P

ratio of 0.25 (compare our diet nos. 3 and 4, fig. 2) Bethke, Kick and Wilder ('32) observed their poorest growth, irrespective of the presence of vitamin D. The weight of young weaned on such diets, as reported here, was exceptionally good. Similarly, our results with a Ca/P ratio of 3.0 (diet no. 11) might be interpreted as relatively better than the growth reported by these authors for a diet with a similar ratio at a low level. To assure the well-being of her young, the lactating mother may be expected to make the maximum

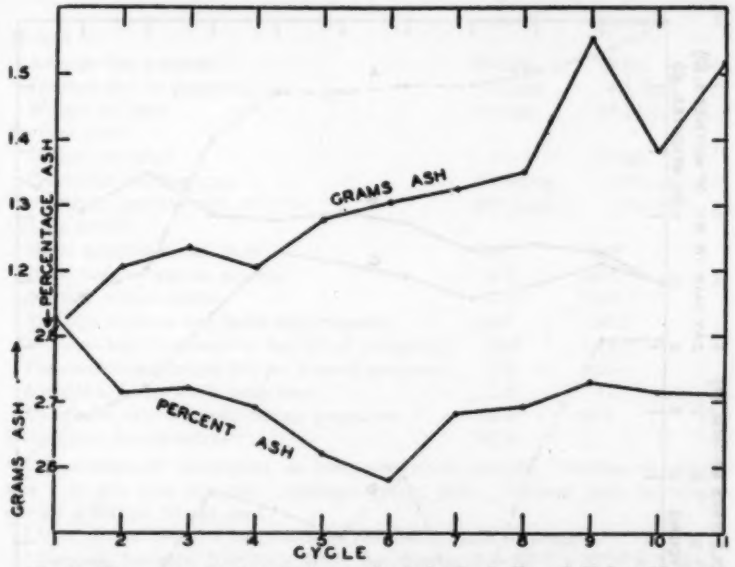

Fig. 6 The per cent ash and grams of ash in the bodies of 21-day-old young raised by the control mothers, plotted for successive cycles.

possible adjustment in her milk. This is indicated by the data of Huffman, Robinson and Winter ('30). We have calculated from their reported data the change in the calcium and phosphorus content of the milk of lactating cows on different levels of mineral intake at different levels of milk production (table 9).

In spite of doubling the mineral intake, the percentage of calcium and phosphorus remained constant. An interesting

TABLE 9

Calculated from the data of Huffman, Robinson and Winter ('30)

MILK PRODUCTION	AVERAGE MILK DAILY	INTAKE		COMPOSITION OF MILK	
		Ca	P	Per cent Ca	Per cent P
	kg.	*gm.*	*gm.*		
Low	8.2	32.3	32.6	0.115	0.090
	11.2	77.9	61.3	0.111	0.090
High	27.3	49.7	57.0	0.109	0.088
	29.5	131.9	104.7	0.116	0.090

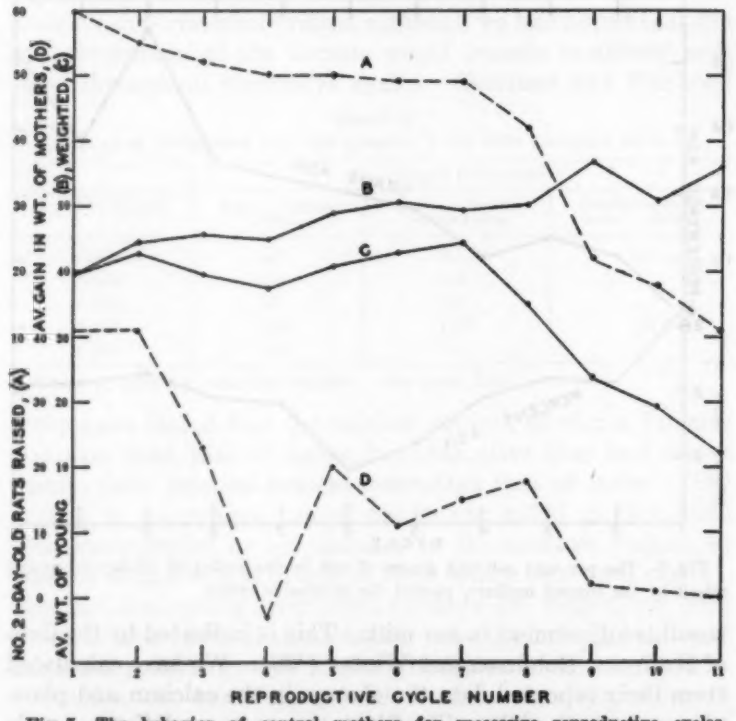

Fig. 7 The behavior of control mothers for successive reproductive cycles. Curve A shows the total number of young raised by these mothers; Curve B, the average weight of these young; Curve C, the corrected weight (corrected for the number that should have been raised) and, Curve D, the average weight fluctuations of the mothers themselves.

problem for further investigation is the determination of the effect of different Ca/P ratios on the Ca/P ratio of the milk.

We have thought it worth while to calculate from the calcium intake of our experimental rats, what would be the equivalent intake by human mothers. We have made such a comparison on the basis of weight, surface area and caloric intake.[9]

[9] Comparison of weight, surface area and caloric intake of woman and rat during reproduction.

	A Rat	B Woman	$\frac{B}{A}$
Weight			
Average non-pregnant	300 gm.	58 kg.	193
Increase due to pregnancy	87 gm.[1]	9.1 kg.[2]	
Weight at term	387 gm.	67.1 kg.	173
Surface area[3]			
Height (in cm.)	...	160 cm.	
Calculated surface area	408 sq.cm.	1.60 sq.m.	39
Calculated surface area at term	485 sq.cm.	1.70 sq.m.	35
Caloric intake			
Basal metabolism cal./sq.m.	766[4]	888[5]	
Basal requirement, in calories	31.3	1421	
Normal caloric intake	57.5[6]	2730[7]	
Per cent increase over basal requirements	183.7	192.1	
Per cent heat increment in last 0.4 of pregnancy	12.5[8]	12.5[8]	
Caloric increase in last 0.4 per cent of pregnancy	3.9	178	
Distributed over whole pregnancy	1.6	71	
Calculated caloric intake during pregnancy	60.4[10]	2865	45
Determined caloric intake[11]	63.9	....	

[1] Experimentally determined on forty-nine stock animals. Increase in weight was 3.22 per cent per pup. Average young, nine. Average gain in weight during gestation 29 per cent.

[2] Many authorities cite 20 pounds as the desired gain in weight.

[3] Formula, for rats, $S = 9.1 \times W^{0.66}$; for women, $S = H^{0.725} \times W^{0.425} \times 71.84$.

[4] Benedict and McLeod, J. Nutrition, vol. 1, p. 373 (1929). Average value for 15-month-old rats in winter and summer at 28°C.

[5] Du Bois, E. F., Basal metabolism in health and disease.

[6] Experimentally determined on 300 gm. resting mother rats.

[7] Average of caloric intakes given by Lusk for housemaids and laundresses at 8-hour hard and medium work, with an additional 10 per cent for fecal loss. See Shukers, C. F., Macy, I. G., Donelson, E., Nims, B., and Hunscher, H. A., J. Nutrition, vol. 4, p. 399 (1931).

[8] Assuming same as for woman.

[9] Calculated from data of Sandford and Wheeler, J. Biol. Chem., vol. 62, p. 329 (1924).

[10] Example: $(31.3 + 1.6) \times 183.7$.

[11] Average of twenty-seven gestations of rats on diet no. 1.

Table 10, based on the calcium intake of rats on diet no. 1, shows that on a weight basis, human mothers would ingest 6.75 gm. calcium daily to equal the intake of such rats during gestation; on a surface area basis, 1.37 gm., and on a caloric basis, 1.75 gm.

Comparisons on the basis of surface area are more dependable than those on weight. If we can tentatively accept 1.37 gm. as the human calcium equivalent of diet no. 1 (calcium level 0.245 per cent, success grade 76.0), then diet no. 7 (calcium level 0.490 per cent, success grade 95.8) is approximately equivalent to 2.74 gm., and diet no. 27 (calcium level,

TABLE 10

Theoretical calcium intake of woman compared to rat on weight, area and caloric basis

COMPARED ON BASIS	INTAKE Ca PER RAT PER DAY (DIET NO. 1)	FACTOR B/A FOOTNOTE 9	THEORETICAL DAILY CALCIUM INTAKE OF WOMAN	
			During gestation	During lactation
	gm.		*gm.*	*gm.*
Weight	0.039	173	6.75	
	0.044	193		8.49
Area	0.039	35	1.37	
	0.044	39		1.72
Caloric intake	0.039	45	1.75	....

0.122 per cent, success grade 38.1), to 0.63 gm. If it were possible to place women under experimental conditions similar to those described here and assure the three mineral intakes mentioned, one might expect curves similar to B, C and D of figure 4. The average calcium intake of American women does not greatly exceed 0.63 gm. (Sherman, '32; Schmidt and Greenberg, '35). On the basis of their balance studies, Toverud and Toverud recommend 1.5 to 2.0 gm. daily, while most authorities feel 1.0 to 1.5 gm. is adequate during gestation. At this low calcium level (0.245 per cent, equivalent to 1.37 gm.), a Ca/P ratio of less than 1.0 would probably be optimal (compare figs. 1 and 2). A ratio less than one is recommended by Sherman ('32) for normal adult maintenance, and Toverud ('33) for gestation. It is not clear why

a ratio less than 1.0 is preferable at low calcium levels. Possibilities that suggest themselves are: 1) the requirement of phosphorus for tissue growth is relatively greater during this period and is in excess of the minimal calcium level at which skeletal growth can proceed, or 2) at ratios greater than 1.0 there is more calcified body tissue.

SUMMARY

1. One hundred and forty-five female rats (who gave birth to 8431 pups, and raised 4391 to 21 days of age) have been studied for ten consecutive reproductive cycles while receiving a diet of purified foodstuffs containing a constant quantity, but varying levels and ratios, of calcium and phosphorus.

2. Both level and ratio of mineral elements exerted an effect on the success of the mothers in producing and rearing young.

3. A Ca/P ratio of 1.0, at a calcium level of 0.490 per cent, was adjudged (on the basis of weight of young at 21 days, ash content of the young, and change in weight of the mothers) the ideal mineral level and ratio for successful gestation and lactation in rats.

4. By employing the average weights of 21-day-old young it was shown that the optimal ratio depends upon, and is proportional to, the calcium level. If the calcium intake is not known exactly, a Ca/P ratio of 1.0 will approximate the optimal.

5. At excessive mineral levels (2.45 per cent) poor performance was obtained, irrespective of ratio.

6. The largest ash contents of the 21-day-old young were uniformly obtained when the mother rat received a diet with Ca/P ratio of 1.0.

7. At constant phosphorus intake (0.245 per cent), increasing the calcium content of the mothers' diet from 0.017 to 0.490 per cent gave better reproductive success; further calcium increases made for rachitic diets and failure.

8. Phosphorus (PO_4) in excess was better tolerated than calcium in excess.

9. The relationship of these findings to human nutrition has been discussed.

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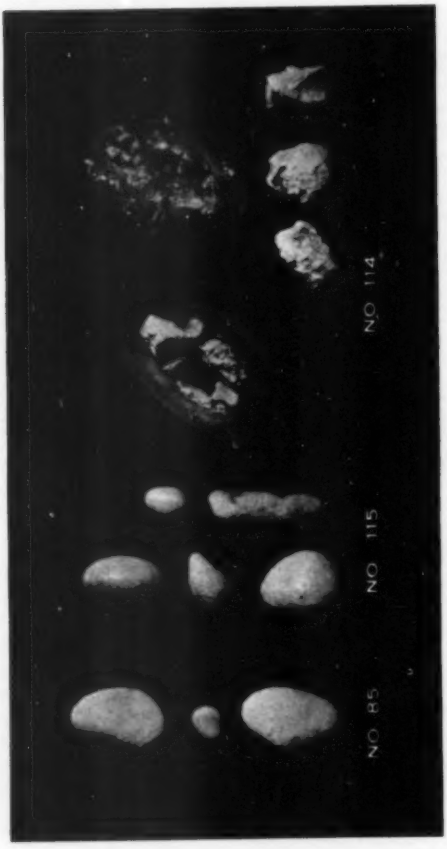
PLATE 1



Photograph showing the actual size of urinary and renal calculi obtained from three rats. Rat no. 85 was on diet no. 17. Rats nos. 114 and 115 were on diet no. 23.

THE MINERAL COMPOSITION OF YOUNG RATS

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FIVE FIGURES

(Received for publication September 18, 1935)

In the preceding paper (Cox and Imboden, '36) we drew attention to the effect of different levels and ratios of calcium and phosphorus in determining reproductive success.[1] During this work we had followed more than 8000 young rats (inbred Wisconsin strain) and had compiled considerable data on the inorganic composition of the young, and the reproductive behavior of mother rats. In the following sections we present these additional data.

Effect of dietary minerals on the composition of the newborn rat. One hundred and forty-five mother rats gave birth to 8431 pups during the course of our work. The young were weighed on the day of their birth (usually they had suckled—as indicated by a white patch across the belly), and 522 of them analyzed for calcium and phosphorus, after ashing the entire rat. In table 1 the composition of first litter pups from mothers which received stock food, is compared with that of pups from mothers on twenty-four experimental diets, containing different mineral levels.

In spite of the fact that there was a great difference in the mineral content of these experimental diets the composition of the pups was within the range observed in analyses of stock rats, and the averages were practically identical (table 1). Certainly it can be said that a change in dietary minerals

[1] The composition of experimental diets referred to in this work is given in the previous paper.

is not immediately reflected by a change in the composition of fetal tissue, and, conversely, that the maternal organism is able to regulate the mineral elements supplied to the fetus.

However, not all mineral levels can be so compensated. Diets excessively high in calcium and unbalanced as regards

TABLE 1

Composition of rat pups

DIET	NUMBER OF ANIMALS	AVERAGE WEIGHT, GRAMS	NUMBER OF DETERMINATIONS	ASH		CALCIUM		PHOSPHORUS		OBSERVER
				Per cent	A.D.[1]	Per cent	A.D.[1]	Per cent	A.D.[1]	
Stock first litter	239	5.37	50	1.754	0.056	0.269	0.017	0.280	0.028	....
Experimental diets, various second litter	263	5.12	53	1.730	0.060	0.269	0.021	0.294	0.011	....
Diets 21 to 23 second litter	20	4.89	6	1.512	0.077	0.195	0.037	0.233	0.016	....
Reports of other investigators										
Stock	166	....	..	....	....	0.260	....	0.295	....	Goss and Schmidt ('30)
Stock	15	4.7	..	....	....	0.250	....	....	....	Sherman and McLeod ('25)
Stock	21	4.3	..	....	....	....	....	0.34	....	Sherman and Quinn ('26)
Various Ca levels	37	4.4	..	....	....	0.28	....	....	....	Sherman and Booher ('31)
Stock	...	....	..	1.576	....	0.271	....	0.267	....	Toverud ('23–'24)

[1] Average deviation.

phosphorus, nos. 21 to 23, did adversely affect the composition of the newly born, as indicated in table 1. Other diets, notably diet 16, Ca/P ratio, 5.0, gave a lowered ash content of the young after several successive reproductive cycles. These young are being examined histologically[2] and it may be said,

[2] By Dr. E. A. Park, Harriet Lane Home, Johns Hopkins University.

in advance of publication, that they show evidence of experimental congenital rickets.

Booher and Hansmann ('31–'32) have noted the constancy of the percentage bone ash in newly born infants, in spite of what was judged to be large differences in maternal intakes of calcium and phosphorus. Similarly, Macomber ('27) has shown that diets low in calcium do not cause a reduction of the calcium content of the rat at birth; he attributes this lack of effect to the relatively low calcium content of the rat fetus. The cartilagenous nature of the rat at birth as compared to the human infant at birth (Booher and Hansmann, '31–'32; White House Conference, '32) is illustrated in table 2.

TABLE 2

The inorganic composition of the rat, and human infant at birth

	WEIGHT	PER CENT ASH	TOTAL WEIGHT		PER KILOGRAM BODY WEIGHT		PER CENT ASH		Ca : P RATIO IN ASH
			Calcium	Phosphorus	Calcium	Phosphorus	Calcium	Phosphorus	
	gm.		*gm.*	*gm.*	*gm.*	*gm.*			
Rat pup	5.37	1.754	0.0144	0.0150	2.68	2.79	15.3	16.0	0.96
Human fetus[1]	3000	3.33	24.21	13.32	8.07	4.44	24.2	13.3	1.82
21-day-old rat[2]	44.7	2.738	0.313	0.236	7.01	5.27	25.7	19.3	1.33

[1] Full term.

[2] Average of 420, 21-day-old stock rats.

Such a comparison indicates that gestation per se causes little strain on the mineral stores of mother rats, in contradistinction to the considerable strain of gestation on human mothers. Thus, the lack of mineral demand during gestation in rats makes the balance experiments of Goss and Schmidt ('30), in which they showed consistently positive calcium balances in rats during pregnancy, more readily understandable. The composition of the rat, in terms of ash, calcium and phosphorus, approaches that of the infant at birth, only after the former has suckled some 21 days.

Body ash at 21 days of age

To establish standards for our rat colony, and for purposes of comparison we have determined the total body ash of 420 stock rats 21 days of age, 570 rats less than this age and twenty-three rats 43 days old. In figure 1 we have plotted the weight of rat against the weight of the ash[3] (the only 21-day-old rats used in this curve were those known to be the first

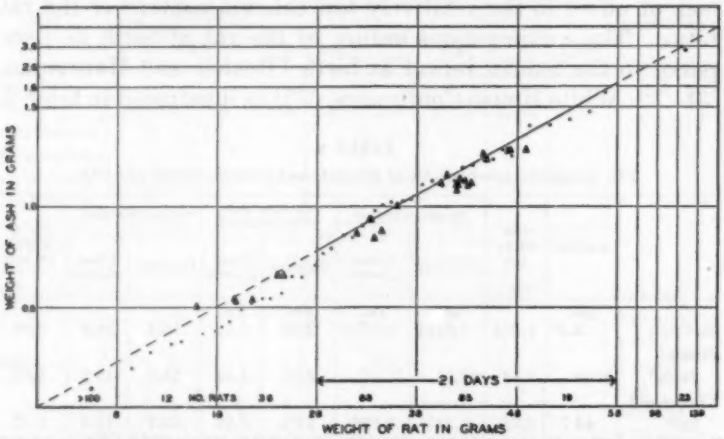

Fig. 1 Weight of rats plotted against the weight of ash. The range of weight of 21-day-old, first litter rats from stock mothers is indicated by an arrow. The small dots indicate averages—averages below 28 gm. indicate rats younger than 21 days. Triangles are averages of 21-day-old rats whose mothers received experimental diets with different amounts of calcium and phosphorus (Cox and Imboden, '36).

young from stock mothers). For 21-day-old rats the average figures gave a straight line, which, when extended, was considerably above the ash content of younger rats, and rats at birth. The triangles are averages of each of the experimental diets (3147 rats were ashed, and the averages used in determining the position of the triangle on the curve) (Cox and

[3] After chloroforming, the rats were weighed on a torsion balance, the gastro-intestinal tract removed, and the carcasses ashed at 550°C. In plotting figure 1, and determining percentages, the gross body weight was used.

Imboden, '36). Most of the values lie fairly close to the control line. It is evident that small rats, 21 days of age, have a larger mineral content than younger rats of the same weight.

The slope of this curve corresponds to an ash percentage of 2.775, and in the evaluation of our experimental rats we employed this value. The actual average of the 420 (various

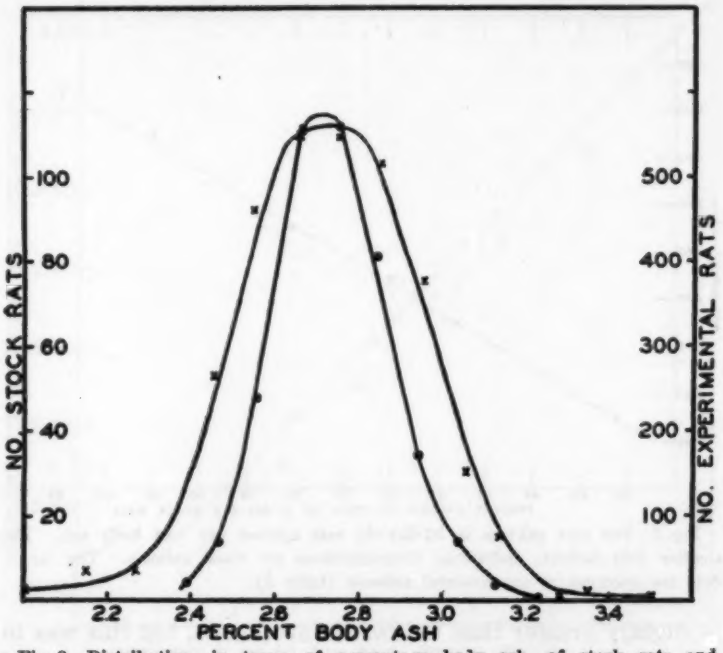

Fig. 2 Distribution, in terms of percentage body ash, of stock rats and experimental animals reported in this work. The curve for experimental animals is indicated by crosses.

litter numbers) 21-day-old rats was 2.738 per cent, and their distribution curve (fig. 2) would indicate that a fair sample had been employed. On the basis of this sample we have determined that an average rat of our stock colony, at 21 days of age, will weigh 44.7 gm. and will contain 1.22 gm. ash (2.738 per cent), 0.701 per cent calcium, and 0.527 per cent

phosphorus. The ash will contain 25.66 per cent calcium and 19.30 per cent phosphorus. There is but very slight variation in the composition of the ash (in calcium $\pm$ 0.44 per cent,[4] in phosphorus $\pm$ 0.37 per cent[4]).

The distribution of the 3147 experimental rats, in terms of percentage ash is given in figure 2. The range of ash content

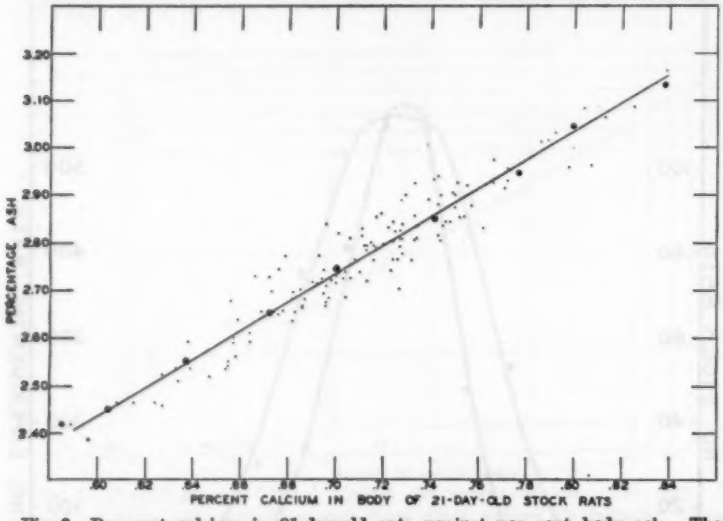

Fig. 3 Per cent calcium in 21-day-old rats against per cent body ash. The smaller dots indicate individual determinations on stock animals. The larger dots are averages of experimental animals (table 3).

is slightly greater than that of the stock rats, but this was to be expected in view of the wide range in mineral content of the diets.

Calcium and phosphorus in 21-day-old rats

Because of the constancy in the composition of the ash it is obvious that the amount of calcium and phosphorus in the bodies of rats is dependent upon the amount of ash. We have illustrated this point in figures 3 and 4, by plotting the

[4] Average deviation.

per cent calcium and phosphorus[5] against the per cent ash. The smaller points indicate single determinations on first litter, 21-day-old stock rats, and the larger points are averages of 544 calcium and phosphorus determinations on ex-

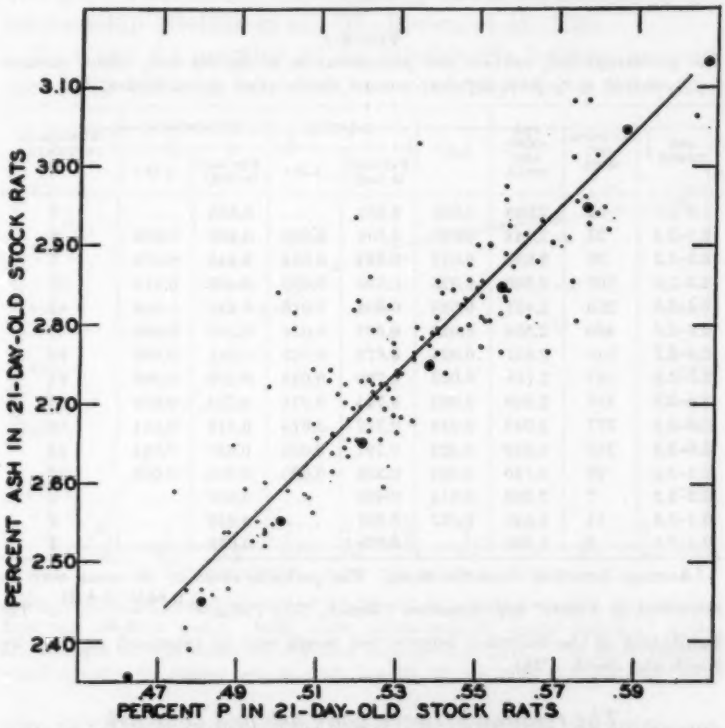

Fig. 4 Per cent phosphorus in 21-day-old rats against per cent body ash. The smaller dots indicate individual determinations on stock animals. The larger dots are averages of experimental animals (table 3).

perimental rats. If the percentage ash is known, the percentage calcium or phosphorus in the body may be read directly from the graph with but little error. The average calcium

[5] Calcium was determined by titration of the oxalate with permanganate—essentially the method of McCrudden ('09–'10), and phosphorus, by the official volumetric method of the A. O. A. C. ('30).

values for the experimental rats agree well with the curve drawn from data on the stock colony, while the values for phosphorus are slightly below those for stock rats.

Table 3 gives in concise form these average values, and the deviations from the average.[6]

TABLE 3

The percentage ash, calcium and phosphorus in 21-day-old rats, whose mothers received diets with different mineral levels (Cox and Imboden, '36)

ASH RANGE	NUMBER OF RATS	PER CENT ASH MEAN	A.D.[1]	CALCIUM		PHOSPHORUS		NUMBER OF DETERMINATIONS
				Per cent in body	A.D.[1]	Per cent in body	A.D.[1]	
1.9–2.1	12	2.008	0.028	0.424	....	0.395	....	2
2.1–2.2	35	2.148	0.022	0.506	0.012	0.422	0.008	8
2.2–2.3	36	2.266	0.019	0.543	0.014	0.445	0.008	7
2.3–2.4	102	2.360	0.025	0.576	0.013	0.463	0.010	28
2.4–2.5	265	2.451	0.028	0.604	0.016	0.481	0.008	48
2.5–2.6	460	2.554	0.023	0.637	0.014	0.502	0.008	81
2.6–2.7	548	2.651	0.024	0.672	0.015	0.521	0.009	95
2.7–2.8	547	2.748	0.022	0.700	0.013	0.538	0.009	91
2.8–2.9	516	2.849	0.023	0.741	0.014	0.556	0.009	81
2.9–3.0	377	2.948	0.024	0.777	0.015	0.578	0.011	58
3.0–3.1	150	3.049	0.023	0.797	0.016	0.587	0.014	28
3.1–3.2	76	3.140	0.021	0.838	0.020	0.608	0.009	12
3.2–3.3	7	3.282	0.013	0.890	....	0.655	....	2
3.3–3.4	11	3.346	0.037	0.907	....	0.649	....	2
3.4–3.5	5	3.499	....	0.939	....	0.672	....	1

[1] Average deviation from the mean. The probable error of the mean may be calculated by Peters' approximation (Munch, '31) $P.E._m = \frac{0.8453 \times A.D.}{\sqrt{n}}$. The significance of the difference between two means may be calculated as given by Smith and Smith ('34).

The relation between body ash and bone ash

It was previously stated (Cox and Imboden, '36) that maternal diets containing different amounts of calcium and phosphorus would cause mother rats to raise young with different percentages of body ash. It has not previously been shown that per cent body ash bears any relation to the commonly accepted measure of calcification; i.e., bone ash. The

[6] We have determined the statistical significance of the differences in per cent ash, calcium and phosphorus. The significance ratios (Smith and Smith, '34) were greatly in excess of 3.0.

effect of Ca : P ratios on calcification has usually been studied by feeding the young, weaned rat, and determining the resultant bone ash. The work in this field has been summarized by Bethke, Steenbock and Nelson ('23–'24). More recently two papers have contributed greatly to our knowledge of this relationship (Bethke et al., '32; Brown et al., '32).

To study the question of the relationship between percentage body ash and bone ash, we routinely, for a time, took

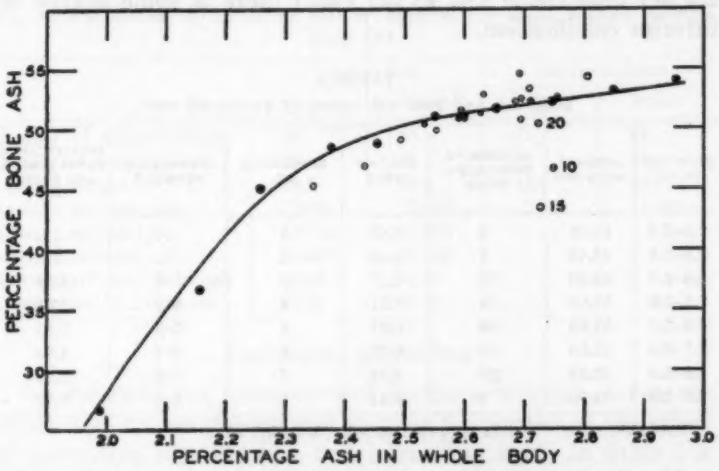

Fig. 5 The per cent body ash of 21-day-old rats plotted against the per cent bone ash of litter mates. Solid black points indicate group averages; circles are averages of rats raised on different experimental diets. The three numbers stated opposite the circles refer to diets nos. 10, 15, etc. (Cox and Imboden, '36).

one rat from each litter raised by mothers on different mineral levels and determined the percentage ash of the dry, alcohol-ether extracted bones (Bethke, Steenbock and Nelson, '23–'24). Both tibiae, humeri and femora were used. The remaining rats in the litter were ashed in an electric muffle after removal of the gastro-intestinal tract. One hundred and fifty-four litters were treated in this way.

When the percentage bone ash was plotted against the percentage body ash of litter mates a smooth curve was obtained. In figure 5 the solid dots indicate group averages;

i.e., as between body ash percentages of 2.5 to 2.6, etc. The differences in bone ash of adjacent points on the more asymptotic part of the curve are not statistically significant, but between alternate points the differences are significant (table 4).

Figure 5 demonstrates that body ash may be used as a measure of bone ash or of the degree of calcification in 21-day-old rats. When the body ash percentage is less than 2.4 per cent (bone ash 48 per cent) there is some degree of inferior calcification.

TABLE 4

Body ash and bone ash values of 21-day-old rats

BODY ASH RANGE	AVERAGE BONE ASH	NUMBER OF DETERMINATIONS	P.E.[1] OF MEAN	REFERENCE NO.	DIFFERENCE BETWEEN	SIGNIFICANCE RATIO (SMITH AND SMITH, '34)
2.2–2.3	45.10	4	0.88	1	...	....
2.3–2.4	48.48	7	0.40	2	...	....
2.4–2.5	48.80	23	0.27	3	3–1	4.51
2.5–2.6	51.00	34	0.21	4	4–2	5.58
2.6–2.7	51.68	36	0.27	5	5–3	7.54
2.7–2.8	52.59	20	0.27	6	6–4	4.64
2.8–2.9	53.22	20	0.38	7	7–5	3.31
2.9–3.0	54.06	8	0.41	8	8–6	2.99

[1] Probable error. Peters' approximation (Munch, '31).

When the determinations were rearranged in accordance with the diet employed (circles), two points were definitely off the curve. These were rats raised on diets nos. 10 and 15 which contained a high level of phosphorus (2.45 per cent) at a Ca/P ratio of 0.2 and 0.3. On such high phosphorus diets the body ash cannot be used as an expression of the degree of calcification. In spite of a normal per cent body ash, the bone ash was sufficiently low to indicate a degree of rickets (high phosphorus rickets). The young were very small (15 to 18 gm.) and we are inclined to attribute the above finding to the concentration of mineral elements in their tissues: an hypothesis not tenable with small rats exhibiting a high calcium rickets.

Calcification of male and female rats

It has been shown that female rats, before reproduction, contain larger amounts of mineral elements than do male rats (Sherman et al., '25, '26, '31; Hammett, '23). We have compiled our figures in which the sex of the rats was recorded, and present the results in table 5. The conclusions of previous investigators are confirmed.

TABLE 5

The mineral content of 21-day-old rats of different sex whose mothers received stock diet

	FIRST LITTER		UNKNOWN LITTER	
	Male	Female	Male	Female
Number of determinations	42	40	31	36
Number of rats	42	49	31	36
Average weight	39.5	39.6	42.9	42.5
Average per cent ash	2.791	2.856	2.783	2.826
Average per cent Ca	0.711	0.740		
Average per cent P	0.537	0.548		
Average per cent Ca in ash	25.46	25.90		
Average per cent P in ash	19.24	19.21		

Successive gestations

We followed ten stock control mothers through eleven reproductive cycles and present in table 6 a record of the effect of successive reproduction and advancing age. In table 7 a similar compilation for the 135 experimental mothers is presented.

Such data on a stock colony (albino) has been previously reported by King ('15, '24), and King and Stotsenburg ('15). A comparison of our results with King's data indicates either the improvement that has taken place in experimental rat colonies, or the wide difference between albino and extracted rats. It is to be especially noted that only 2 per cent of her albino and 21 per cent of her piebald females produced an eleventh litter, whereas 40 per cent of our controls successfully cast an eleventh litter. Gray Norway rats had only an average 3.69 litters during their reproductive life (King and Donaldson, '29). Even on diets of purified foodstuffs (and

various mineral levels) our rats exhibited better average reproduction than King's stock animals.

These tables justify, we feel, the following generalizations: 1) the number of young born is a function of the cycle

TABLE 6

Reproductive record of stock rats for eleven successive cycles

CYCLE NO.	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	AVERAGE NUMBER PER MOTHER	AVERAGE WEIGHT, GRAMS	NUMBER OF YOUNG AVAILABLE TO RAISE	NUMBER OF YOUNG RAISED TO 21 DAYS	PER CENT RAISED	AVERAGE WEIGHT, GRAMS
1	10	98	9.8	5.76	60	60	100	39.19
2	10	93	9.3	5.67	57	55	96.5	44.45
3	10	107	10.7	5.40	60	52	86.7	45.51
4	10	100	10.0	5.66	60	50	83.3	44.72
5	9	83	9.2	5.63	54	50	92.6	48.83
6	9	74	8.2	5.55	52	49	94.2	50.59
7	9	70	7.8	5.55	50	49	98.0	49.44
8	8	71	8.9	5.38	48	42	87.5	50.20
9	7	45	6.4	5.31	37	22	59.5	56.91
10	5	37	7.4	5.42	26	18	69.2	50.97
11	4	23	5.7	5.17	20	11	55.0	56.00

TABLE 7

Reproductive record of experimental rats for eleven successive cycles

CYCLE NO.	NUMBER OF LITTERS	NUMBER OF YOUNG BORN	AVERAGE NUMBER PER MOTHER	AVERAGE WEIGHT, GRAMS	NUMBER OF YOUNG AVAILABLE TO RAISE	NUMBER OF YOUNG RAISED TO 21 DAYS	PER CENT RAISED	AVERAGE WEIGHT, GRAMS
1'	135	1388	10.3	5.62	810	800	98.8	40.92
2	128	1253	9.8	5.30	753	548	72.8	36.08
3	119	1040	8.7	5.32	687	504	73.4	36.10
4	112	954	8.5	5.30	645	485	75.2	35.05
5	101	820	8.1	5.18	567	411	72.5	38.66
6	87	635	7.3	5.32	467	323	69.2	40.63
7	74	505	6.8	5.37	374	248	66.3	41.90
8	58	363	6.3	5.36	286	204	71.3	42.79
9	46	265	5.8	5.55	218	155	71.1	45.80
10	42	239	5.7	5.50	199	158	79.4	42.74
11	31	168	5.4	5.32	141	97	68.8	44.10

[1] Stock food.

number; 2) the average weight of rat pups soon after birth is fairly constant;[7] 3) heavier rats are raised as the average number per litter decreases.

[7] We cannot take issue with the finding of King ('24), that the weight of newborn rats is a function of the age of the mother inasmuch as our average weights usually include the weight of milk from a first nursing.

SUMMARY

Data on ten stock and 135 experimental rats, receiving different levels and ratios of calcium and phosphorus, and 8431 young born to these mothers during eleven consecutive reproductive cycles have been compiled. It is noted that: 1) the rat is relatively cartilagenous at birth; 2) the mineral content of the rat at birth is relatively constant, in spite of wide differences in maternal mineral intake; 3) because of constancy in the composition of the body ash of 21-day-old rats, even when their mothers receive varying mineral intakes, the percentage of calcium and phosphorus in their gross bodies is calculable with fair accuracy from the percentage ash; 4) with the exception of high phosphorus diets, the body ash of sucklings (21 days old) may be used as a measure of bone ash, or calcification; 5) female rats, 21 days old, contain more ash, calcium, and phosphorus than do males of the same age; and 6) during consecutive reproductive cycles the number of young born per litter decreases, but the average weight at weaning increases.

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ERRATUM

Volume 11, page 135. Footnote 1 should read as follows:

Working under a grant from the California Fruit Growers Exchange.